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A study of the hexose transport in the rat adipocyte.

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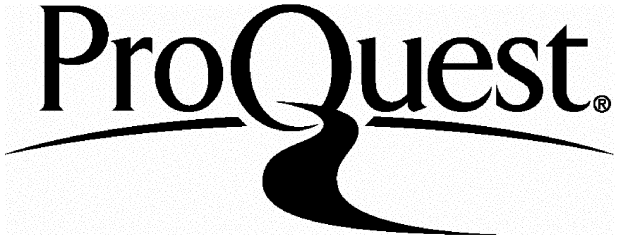


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Summary

The insulin sensitive hexose transport system of the isolated rat adipocyte has been studied. 3-O-methyl-D-glucose, D-allose and D-xylose are all transported by the hexose transport system.

By studying the inhibition of D-allose transport by a range of hexose analogues the hydrogen bonding and spatial requirements of the transporter have been investigated. The transporter accepts the hexose molecule in a pyranose ring form. The results indicate that the important hydrogen bonding positions are the ring oxygen, C-1 and C-3. There may be a weaker hydrogen bond to C-6. Sugar hydroxyls in non-glucose configurations may sterically hinder transport. The inhibition by alkyl substituted hexoses indicates that the hexose transporter requires a specific orientation of the hexose molecule as it approaches the external binding site. C-1 faces the transporter whilst C-4 faces the external solution. When the hexose molecule is bound to the hexose transporter there is little space around C-1 and C-2. There is more space around C-3, C-4 and C-6 with the possibility of a hydrophobic region adjacent to C-6.

4,6-O-ethylidene-D-glucose and alkyl-β-D-glucosides enter the adipocyte independently of the hexose transport system. The results indicate that 4,6-O-ethylidene-D-glucose is a good side specific analogue with a high affinity for the external site. In contrast alkyl-β-D-glucosides are good side specific inhibitors on the inside of the hexose transport system. Both 4,6-O-ethylidene-D-glucose and n'-propyl-β-D-glucoside are competitive inhibitors of the hexose transport system.

Treatment of basal adipocytes with 10nM insulin can lead to a 50-fold stimulation of hexose transport. The hydrogen bonding and spatial requirements of the hexose transporter are unchanged by insulin.

D-glucose and 2-deoxy-D-glucose showed reduced inhibition of D-allose transport when the intracellular ATP levels were depleted by cyanide poisoning. There was no effect of cyanide on the inhibition of transport by 3-O-methyl-D-glucose. This result suggests a possible effect of hexose metabolites on the hexose transporter.

A range of purine and pyrimidine nucleosides, nucleotides and cyclic nucleotides were tested for possible effects on hexose transport when added exogenously to adipocyte suspensions. Adenosine (10μM) and inosine (0.1mM) were found to give small stimulations of hexose transport in basal adipocytes. Xanthine stimulated hexose transport in basal adipocytes and inhibited transport in insulin-stimulated cells. Exogenous ATP inhibited hexose transport in insulin-stimulated cells. Cyclic nucleotides did not affect the rate of hexose transport in adipocytes.

Abbreviations

AMP -	Adenosine-5'-monophosphoric acid
2'(3') AMP -	Adenosine 2'(3')-monophosphoric acid (mixed isomer)
ADP -	Adenosine-5'-diphosphate
ATP -	Adenosine-5'-triphosphate
8-bromo-cyclic AMP -	8-bromoadenosine-3':5'-cyclic monophosphoric acid
8-bromo-cyclic GMP -	8-bromoguanosine-3':5'-cyclic monophosphoric acid
8-bromo-cyclic IMP -	8-bromoinosine-3':5'-cyclic monophosphoric acid
cyclic AMP -	Adenosine-3':5'-cyclic monophosphoric acid
cyclic GMP -	Guanosine-3':5'-cyclic monophosphoric acid
dibutyryl cyclic AMP -	N^6-$O^{2'}$-dibutyryl adenosine-3':5'-cyclic monophosphoric acid
EDTA -	Ethylenediaminetetraacetic acid
EGTA -	Ethyleneglycol-bis(β-amino-ethyl ether) N, N' tetraacetic acid
FDNB -	1-fluoro-2,4-dinitrobenzene
GMP -	guanosine-5'-monophosphoric acid
Hepes -	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
NBMI -	6[(4-nitrobenzyl)-thio]-9-β-D-ribofuranosyl purine
NAD -	β-nicotinamide adenine dinucleotide
NADH -	β-nicotinamide adenine dinucleotide (reduced form)
SITS -	4-acetamido-4'-isothiocyano-stilbene-2'-sulphonic acid
Tris -	Tris (hydroxymethyl) amino methane

4,6-O-ethylidene-D-glucose - 4,6-O-ethylidene-D-glucopyranose

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INTRODUCTION

Introduction

The membrane surrounding a cell provides a barrier separating the cell contents from the external environment allowing the cell to maintain a constant internal environment. This barrier cannot be totally impermeable and arrangements must be made to allow for the controlled passage of substrates and waste products through the membrane.

The structure of biological membranes

The study of the composition of biological membranes from a wide variety of sources show membranes to contain approximately 40% of their dry weight as lipid and 60% as protein. There is also a small percentage of carbohydrate present. In addition, native membranes contain approximately 20% of their total weight as water which is tightly bound and essential for the maintenance of membrane structure.

Phospholipids isolated from biological membranes are polar lipids with a hydrophobic tail group and a hydrophilic head group. Most models for membrane structure incorporate a lipid bilayer arrangement where the aliphatic chains are sequestered within the interior of the membrane and the hydrophilic heads face the aqueous solution. This structure is maintained by entropic factors rather than specific hydrophobic interactions.

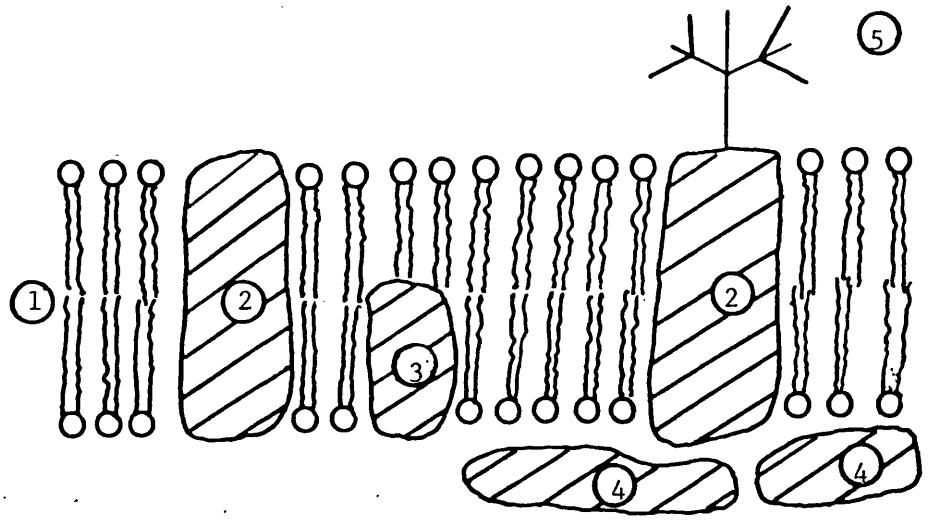
In addition to glycerol based phospholipids, sphingamine derivatives and sterol derivatives are also present. The properties of the membrane, for example the fluidity of the hydrophobic core, are determined by the ratio of these components in the membrane.

Current views on the structure of biological membranes are based on the fluid mosaic model of Singer & Nicolson (1972). In this model globular proteins are embedded in a lipid bilayer. Some of these proteins are able to span the membrane and are accessible at both faces. In this model the hydrophilic portions of both lipids and proteins are in contact with the aqueous surroundings whilst the non polar portions are sequestered in the interior of the membrane away from water. A schematic representation of the fluid mosaic model is shown in Fig. 1.

In addition to the closely associated intrinsic protein molecules, which are embedded in the membrane and interact with hydrophobic regions, other protein molecules are believed to be associated with the polar head groups of the lipid bilayer. It is believed that these extrinsic proteins are attached to the membrane by electrostatic interactions. Thus some membrane proteins can be removed by increases in ionic strength or enzymic removal of polar head groups, but a large proportion of the membrane proteins are intimately associated with the membrane and can only be removed by complete disruption of the membrane by detergents.

At physiological temperatures the hydrocarbon chains of the lipids are mobile within the bilayer, whereas at lower temperatures the hydrocarbon chains form semi crystalline regions which give the membrane gel-like properties. Thus, at physiological temperatures, intrinsic proteins are free to move in the plane of the membrane. The lateral diffusion coefficient of intrinsic membrane proteins has been estimated as 2×10^{-10} cm^2 sec^{-1} which is slower than that of phospholipids (2×10^{-8} cm^2 sec^{-1}) (Harrison & Lunt, 1975). There is, however, considerable

Fig. 1. The Singer Nicolson fluid mosaic model for the structure of biological membranes



A schematic representation of a section through a biological membrane to illustrate the main features:-

(1) Phospholipid molecules forming bilayer

(2) Intrinsic membrane proteins spanning bilayer

(3) Intrinsic protein molecule which is accessible from one face only.

(4) Extrinsic membrane proteins associated with both phospholipids and intrinsic proteins.

(5) Carbohydrate residues on an intrinsic membrane protein. These residues are usually found on the outer face of the membrane.

variation in the rate of lateral diffusion of different proteins within a membrane, an important factor being association with other proteins in the membrane. There is, for example, evidence that the intrinsic band III glycoprotein of the erythrocyte membrane is associated with the extrinsic protein spectrin. In some cell types it is important that the lateral diffusion of protein molecules is controlled; intestinal cells, for example, segregate two different hexose transport activities at opposite ends of the cell.

Facilitated diffusion

The passage of a solute through the cell membrane requires it to enter the hydrophobic lipid region of the membrane and pass through it in order to enter the solution on the opposite side. Observations of biological transport processes indicate that specific substrates are transported in a manner markedly different from simple diffusion through the lipid bilayer. Danielli (1954) named this process for the energy independent transport of selected compounds 'facilitated diffusion'.

Facilitated diffusion systems for the transport of a range of substrates are present in most mammalian cell types. All facilitated diffusion systems utilise an existing electrochemical gradient bringing about transfer of solute from regions of high concentration to low. Facilitated diffusion systems, including the adipocyte and erythrocyte hexose transport systems, show the following features:-

(1) The penetration rate of specific substrates into cells containing a facilitated diffusion system is considerably faster than the rate calculated on the basis of the number of hydrogen bonding groups present on the substrate molecule. For example,

D-glucose penetrates human and rabbit erythrocytes considerably faster than would be expected for penetration by simple diffusion (Laris, 1958). This rate is also faster than that calculated for diffusion through non specific aqueous channels in the membrane (Sha'afi et al., 1971).

(2) Facilitated diffusion systems are stereospecific for their substrates. For example L-glucose penetrates the erythrocyte membrane very slowly when compared to D-glucose. Simple diffusion systems would be expected to be unable to distinguish between stereoisomers, giving similar rates for both isomers.

(3) The rate of penetration of a substrate through a facilitated diffusion system is not directly proportional to the concentration as predicted by Ficks law of diffusion (Fick, 1855). Instead the rate reaches a limiting rate as the substrate concentration is raised (Le Fevre, 1954, and Widdas, 1954). This observation suggests that the substrate interacts with a limited number of membrane sites responsible for transport. Simple kinetic analysis of the erythrocyte hexose transport system and other facilitated diffusion systems show the flux (u) to be given by an equation of the form:

$$u = \frac{S \cdot Vmax}{Km + S} \qquad (1)$$

Where S is the substrate concentration, Vmax and Km are constants unique to any transporter/substrate combination, Vmax is the maximal velocity of transfer and Km the substrate concentration at which half maximal velocity is achieved. Both Km and Vmax are analagous to the Michaelis Menten constants for an enzymic reaction.

(4) The rate of facilitated diffusion is competitively inhibited by molecules which possess a similar structure to the substrate. Competitive inhibition of hexose transport in the erythrocyte was first established by Le Fevre & Davies (1951).

(5) Non-competitive inhibition of penetration has also been observed with molecules which interact irreversibly with the membrane. Mercuric chloride irreversibly inhibits hexose transport in erythrocytes under conditions where no gross morphological change in the membrane is caused (Van Steveninck et al., 1965).

(6) Under certain circumstances it is possible to link the movement of one substrate down its electrochemical gradient with the movement of a structurally similar molecule which is driven up its electrochemical gradient. This process of counterflow was first described by Rosenberg & Wilbrandt (1957) for the uphill transport of D-glucose in the presence of a gradient of D-mannose or unlabelled D-glucose across the erythrocyte membrane. This experiment indicates the presence of sugar binding sites at both surfaces of the transport system; either one facing inward with the other facing outward, or a single site which is alternately available at the inner and outer surfaces.

Kinetic measurements of facilitated diffusion

A number of different experimental protocols are available to study the kinetics of facilitated diffusion. Eilam & Stein (1973) described a nomenclature for such protocols. The transport of a substrate from one face of the membrane (cis) to the opposite face of the membrane (trans) is followed. The most commonly used method of measuring transport rates is to follow the flux of a radiolabelled substrate into (entry) or out of the cell (exit). In order to measure unidirectional flux it is necessary to measure initial rates of transport. The initial rate of transport can be determined either by the use of very short uptake periods or by the use of integrated rate equations which compensate for the backflux that occurs with longer uptakes.

Zero trans experiments. In these experiments the initial rate of flux of substrate is measured when there is no substrate at the opposite face of the membrane. These experiments give the Km and Vmax for influx and efflux.

Equilibrium exchange. In these experiments the initial rates of unidirectional flux of substrate are measured when the substrate concentration is same on both sides of the membrane. Under these conditions there is no net transfer of substrate and the rates of entry and exit are therefore equal for a non-accumulating system. These experiments give the Km and Vmax for equilibrium exchange.

Infinite cis experiments. In these experiments the net flux is measured when the substrate concentration on the cis face of the membrane (outside for infinite cis entry, inside for infinite cis exit) is at a saturating concentration, which is at least 10 times the zero trans Km. Infinite cis

experiments measure the Km for the trans site. Sen & Widdas (1960a) described a method for performing infinite cis exit experiments in erythrocytes using an osmotic swelling method. Cells were loaded with a high concentration of sugar and the rate of efflux into solutions containing low concentrations of sugar was followed. With this method the substrate concentration which gives half maximal inhibition of efflux is equivalent to the Km for backflux using the external site.

Infinite trans experiments. In these experiments the substrate concentration at the trans face of the membrane (inside for infinite trans entry, outside for infinite trans exit) is at a saturating concentration. The rate of net flux from the cis face is then measured. This protocol is equivalent to counterflow experiments and measures the Km at the cis face of the membrane.

Hexose transport

A facilitated diffusion system for the transport of hexoses is present in the plasma membrane of most mammalian cell types. In addition to facilitated diffusion processes the intestine and kidney also show the phenomenon of active transport. In these tissues D-glucose is transported against its concentration gradient.

The measurement of hexose transport is complicated by the difficulty of preparing isolated homogeneous cell populations and by the rapid metabolism of D-glucose when it enters the cell. These technical difficulties have been overcome in some tissues by the development of isolated cell techniques and the use of non-metabolised D-glucose analogues.

Table 1. Kinetic parameters for hexose transport in mammalian cell types

	Human erythrocyte		Rat thymocyte(8)		Rat adipocyte (9)				Rat hepatocyte(10)	
Substrate	D-glucose		3.0.methyl D-glucose		3.0.methyl D-glucose				3.0.methyl D-glucose	
Experiment	Km(mM)	Vmax(mMmin^{-1})	Km(mM)	Vmax(mMmin^{-1})	Km(mM) Basal	Vmax(mMmin^{-1}) Basal	Km(mM) + 10 nM insulin	Vmax(mMmin^{-1}) + 10 nM insulin	Km(mM)	Vmax(mMmin^{-1})
Zero trans entry	1.6 (1)	36.1	7.7	0.64	5.41	2.04	6.10	72.0	20.5	82.3
Zero trans exit	25.0 (2)	129.0	-	-	4.09	9.18	2.66	71.4	21.6	91.5
Equilibrium exchange	34 (3)	360	25	2.1	4.22	3.48	4.45	50.4	19.0	87.9
Infinite cis entry	2.8 (4)	-	-	-	9.03	3.96	6.51	58.8	-	-
Infinite cis exit	1.8 (5)	-	-	-	3.60	6.36	3.60	105.6	-	-
Infinite trans entry	1.7 (6)	174	~25+	0.14+	-	-	-	-	-	-
Infinite trans exit	3.4 (7)	17*	-	-	-	-	-	-	-	-

References

1. Lacko et al. (1972)
2. Karlish et al. (1972)
3. Naftalin & Holman (1977)
4. Hankin et al. (1972)
5. Sen & Widdas (1962)
6. Ginsburg & Stein (1975)
7. Eilam (1975)
8. Whitesell et al. (1977)
9. Taylor & Holman (1981)
10. Craik & Elliot (1979)

Notes

* experiment performed at 2°C
\+ internal concentration 21mM therefore not true infinite trans conditions

Hexose transport in the rat adipocyte

The technical difficulties associated with the study of hexose transport in the rat adipocyte have only recently been overcome. Many of the earlier characterisations of hexose transport in adipose tissue have relied on indirect measurements of sugar uptake such as the rate of glucose oxidation. Crofford & Renold (1965) first reported the presence of a facilitated diffusion system in whole adipose tissue.

Rodbell (1961) has described a method for the preparation of isolated adipocytes, in which the collagen network surrounding the cells was broken down by the action of a bacterial collagenase preparation. This method releases viable cells which respond to hormonal stimulation in a similar manner to intact tissue (Gliemann, 1967). The adipocyte contains a large lipid droplet within the cell which gives adipocytes a low density compared to other cell types. This difference in densities allows adipocytes to be prepared as a homogeneous population free from contamination by other cell types.

Since D-glucose entering the adipocyte is rapidly metabolised it is impossible to directly study the transport of D-glucose. Since the transport of D-glucose is rate limiting for metabolism when the external D-glucose concentration is below 1-2mM (Gliemann, 1968) transport can be followed by indirect methods such as the rate of D-glucose oxidation. The movement of molecules through the membrane transport system can be studied independently of metabolism by the use of D-glucose analogues which are transported but not metabolised.

The D-glucose epimer, D-allose, has been shown by Loten *et al.* (1976) to be transported by adipocytes but not metabolised. The transport of D-allose is much slower than D-glucose due to the high Km of D-allose (> 150mM). Loten *et al.* showed D-allose transport to be com-

petitively inhibited by D-glucose and also demonstrated counterflow in the presence of a D-glucose gradient. They also showed that the rate of D-allose transport was increased in the presence of insulin.

Foley et al. (1978) studied the transport of L-arabinose in the adipocyte. L-arabinose is a D-galactose analogue lacking the C-5 hydroxymethyl group and is therefore non-metabolised. Their results showed that L-arabinose was transported by the hexose transport system and, like D-allose, L-arabinose is transported slowly due to its high Km (> 50mM). Foley et al. also showed L-arabinose transport to be insulin sensitive and competitively inhibited by D-glucose.

C-3 hydroxymethylation of D-glucose to give 3-O-methyl-D-glucose does not affect the transport of the molecule by the hexose transport system but does prevent phosphorylation by hexokinase. When whole cells are incubated with 3-O-methyl-D-glucose there is little or no conversion to other compounds (Csáky & Wilson, 1956). Olefsky (1978) has shown that at equilibrium 3-O-methyl-D-glucose is in free solution within the cell water and in equilibrium with the suspending buffer. The high ratio of surface area to cytoplasmic volume in adipocytes relative to other cell types (Angel & Farkas, 1970) leads to a rapid equilibration of 3-O-methyl-D-glucose between the suspending medium and cytoplasm. This leads to very rapid rates of uptake which have hindered study of transport. Recently Whitesell & Gliemann (1979) developed a new procedure for measuring the uptake of 3-O-methyl-D-glucose. They used the hexose transport inhibitor phloretin to block transport after short time intervals, followed by separation of the cells from the bulk medium by rapid centrifugation through light oil (Gliemann et al., 1972). The results of Whitesell & Gliemann were similar to those of Taylor & Holman (1981)

shown in Table 1. From this kinetic analysis it can be seen that the transport of 3-O-methyl-D-glucose is symmetrical with equal kinetic parameters for all the experimental protocols used. The rate of 3-O-methyl-D-glucose transport is markedly increased in the presence of insulin and a number of investigators have reported that this change is due to an increase in the Vmax rather than a change in the Km (Czech (1976a); Olefsky (1978);Whitesell & Gliemann (1979); Siegel & Olefsky (1980) and Taylor & Holman (1981)).

The transported D-glucose analogue 2-deoxy-D-glucose is a substrate for hexokinase (Olefsky, 1978) and on entering the cell is rapidly phosphorylated to form 2-deoxy-D-glucose-6-phosphate. The phosphorylated sugar is not further metabolised and, since it cannot pass through the membrane is trapped within the cell. Thus 2-deoxy-D-glucose has been widely used as a means of measuring net uptake. Caution must however be used when interpreting the effects of these experiments as long incubations lead to depletion of the intracellular ATP with the result that the transport rate exceeds the rate of phosphorylation. This allows backflux of 2-deoxy-D-glucose to occur. Foley et al. (1980b) have shown that the phosphorylation of 2-deoxy-D-glucose may become the rate limiting step for uptake under some circumstances. Thus the total uptake of 2-deoxy-D-glucose may not give a true indication of the transport rate.

A detailed study of the specificity of the adipocyte hexose transport system has not been previously performed. One of the aims of the study presented in this thesis is to examine the specificity of the adipocyte hexose transporter and to relate the results of this study to the hydrogen bonding and spatial requirements for binding to the hexose transport system. Also presented in this thesis is a study of the

spatial requirements for the binding of hexoses to the hexose transporter of the adipocyte.

Hexose transport in the adipocyte is markedly stimulated by insulin and a number of other compounds. The proposed mechanisms for the regulation of hexose transport in the adipocyte will be discussed later (see p. 55).

Hexose transport in erythrocytes

The fetal erythrocytes of many mammals possess a facilitated diffusion system for monosaccharides but in many species this activity declines rapidly after birth (Widdas, 1955, 1980; Aubby and Widdas, 1980). In primates, however, the transport activity of erythrocytes is not lost after birth. Human erythrocytes are easily prepared in large quantities, and since the rate of D-glucose metabolism is low these cells provide an ideal system for the study of transport.

Kinetic studies of hexose transport in human erythrocytes have been carried out over many years using a full range of experimental methods. A summary of the results of kinetic analysis of D-glucose transport in erythrocytes is presented in Table 1. A similar pattern of kinetic parameters is shown with other transported hexoses, for example D-galactose (Ginsberg & Stein, 1975). The important features of kinetic analysis of human erythrocyte hexose transport are that zero trans experiments show asymmetric rates of transport. Thus zero-trans entry has a low Km and low Vmax whilst zero trans exit has a high Km and Vmax. The rate of equilibrium exchange is rapid with a high Km and Vmax. In contrast the infinite trans and infinite cis experiments both show symmetrical low Km values for both sides of the membrane.

The temperature dependence of sugar transport in human erythrocytes was studied by Sen & Widdas (1960b and 1962a) and by Lacko et al. (1972). These results show the interaction of hexoses with the transporter to have a high activation energy atypical of free solution diffusion (Lieb & Stein, 1972). This is consistent with the formation of several hydrogen bonds between the sugar and the transporter. Zala et al. (1974) measured the enthalpy of the specific interaction of D-glucose with the erythrocyte membrane and concluded that interactions other than D-glucose binding were involved. From their results Zala et al. inferred that a conformational change in the membrane occurred during hexose binding. Lacko et al. (1972) also showed that the change in activation energy for equilibrium exchange and infinite trans experiments was approximately half that for net entry. The temperature coefficient for net exit (Sen & Widdas 1962a) was much less than that for net influx (Lacko et al. 1972). and these differences indicate that exchange is a different process from net flux. As the temperature is lowered the asymmetry of hexose transport in the human erythrocyte increases.

Additional evidence for exchange being a different process comes from the observation of changes in the pH dependence of transport. Bloch (1974) showed that the pH dependence of net flux differs from that for infinite trans flux. Le Fevre (1963) showed that there is no rapid exchange of low affinity sugars such as D-ribose or L-sorbose and, this suggests that high affinity sugars induce changes within the membrane which are not induced by low affinity sugars.

The substrate specificity of the human erythrocyte hexose transport system

Le Fevre & Marshall (1958) first studied the substrate specificity of the hexose transport system of the human erythrocyte. Their results showed that the transport system accepted hexoses in the $^4C_1$ ring conformation. Hexoses in the $^1C_4$ conformation had greatly reduced affinity for the transport system and Le Fevre & Marshall reported decreasing affinity as the relative stability of the $^4C_1$ conformation was reduced.

The hydrogen bonding requirements for the interaction of D-glucose with the human erythrocyte hexose transporter were investigated by Kahlenberg & Dolansky (1972) who studied the inhibition of D-glucose uptake by D-glucose analogues. Kahlenberg & Dolansky also studied the inhibition of D-glucose binding to erythrocyte membranes by D-glucose analogues. In a similar study Barnett et al. (1973a) measured the inhibition constants of D-glucose analogues by the inhibition of L-sorbose entry into human erythrocytes. Both groups proposed that hydrogen bonds were directed towards the hydroxyls at C-1 and C-3. Barnett et al. observed that fluorine substituted hexoses showed a higher affinity than the corresponding deoxy sugars and proposed that since the fluorine accepted a hydrogen bond in a similar way to the oxygen of the hydroxyl, hydrogen bonds were directed towards the oxygen of the hydroxyl. Kahlenberg & Dolansky also proposed that hydrogen bonds were directed towards the ring oxygen and the C-4 hydroxyl. The results of Barnett et al. indicated that the hydrogen bond to the C-4 hydroxyl could also interact with the C-6 hydroxyl. Overall these results show that when D-glucose interacts with the hexose transport system of the human erythrocyte, hydrogen bonds are at some stage during transport directed towards the ring oxygen C-1, C-3, C-4 and C-6 but

that none of these hydrogen bonds is essential for transport. Lacko & Burger (1962) studied the interaction of disaccharides with the human erythrocyte hexose transport system. They observed that non-reducing disaccharides did not inhibit the transport of hexoses. In contrast the reducing disaccharides maltose and cellobiose inhibited hexose transport but were not transported into the cell. Lacko & Burger also reported that methyl glucoside did not inhibit hexose transport and concluded that the interaction of hexoses with the transport system occurred via the reducing residue of the disaccharide.

Baker & Widdas (1973a) reported that the glucose analogue 4,6-O-ethylidene D-glucose was an inhibitor of the erythrocyte hexose transport system. The rate of 4,6-O-ethylidene-D-glucose entry was much slower than would have been expected from its inhibition constant and their results suggested that 4,6-O-ethylidene-D-glucose entered the cell by a non-mediated route. Baker & Widdas (1973b) found that 4,6-O-ethylidene-D-glucose was much less effective as an inhibitor inside the cell than it was outside. Thus purified 4,6-O-ethylidene-D-glucose has an asymmetry of affinities as high as 60-fold (Baker et al., 1978). 4,6-O-ethylidene-D-glucose is also an asymmetric inhibitor of hexose transport in the erythrocytes of fetal and new born guinea pigs (Aubby & Widdas, 1980).

Barnett et al. (1973b, 1975) showed that alkyl derivatives of D-glucose also entered the cell by a non-mediated route. 6-O-alkyl derivatives were found to be inhibitors at the outside of the cell whereas n'-propyl-β-D-glucoside was a more effective inhibitor inside the cell than outside. From their results Barnett et al. (1973b, 1975) proposed that a glucose molecule approaching from the outside was bound to the active site of the transporter by the C-1 end of the molecule. The transporter was then proposed to undergo a conformational

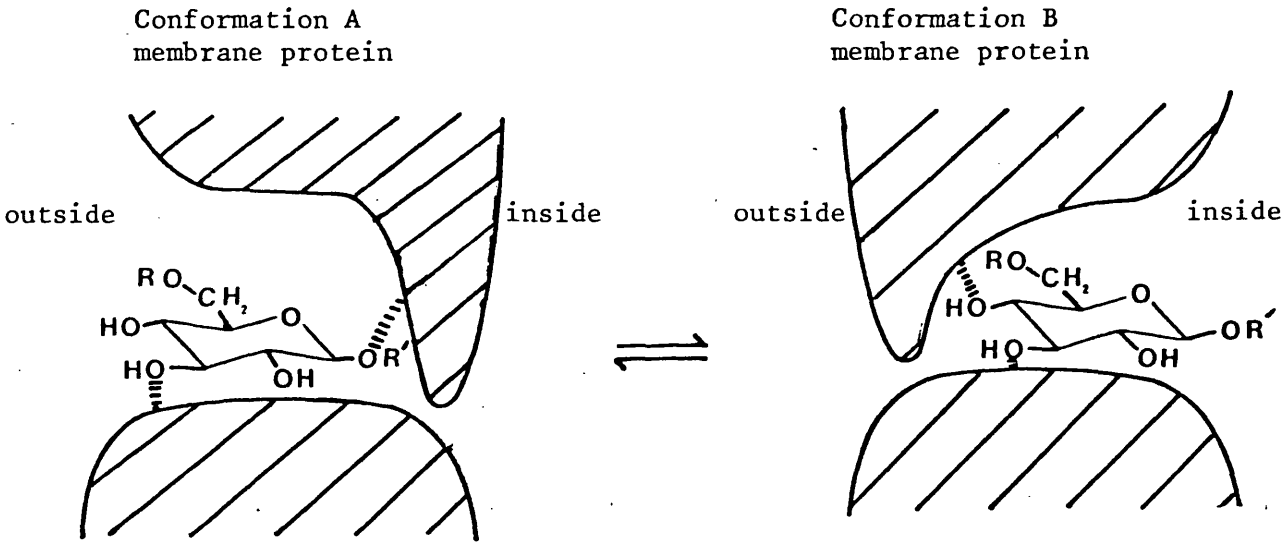
change and the glucose molecule is transferred to the inner site with C-1 facing the internal solution (see Fig. 2).

Evidence for two spatially different sites is consistent with the data of Kahlenberg & Dolansky (1972) who showed that methyl glucosides inhibited glucose binding to erythrocyte membrane fragments, although these compounds did not inhibit glucose transport in whole cells. Kahlenberg & Dolansky also showed phenyl-β-D-glucoside to be a non-competitive inhibitor of D-glucose uptake, an observation consistent with the ability of C-1 modified hexoses to bind to the inside site.

In contrast to these results Novak & Le Fevre (1974) reported that hexoses with alkyl substitutions at both ends of the molecule were inhibitors of hexose transport. On the basis of the results of Barnett et al. (1973b, 1975) these compounds would not be expected to bind to either site. Baker et al. (1978) reported that 1,2-isopropylidene-D-glucose was an asymmetric inhibitor of hexose transport in the erythrocyte with a higher affinity for the outside site (Ki 59mM) compared to the inside site (Ki 74mM). It has also been suggested (Widdas, 1980) that methyl-2-3-di-O-methyl-α-D-glucopyranoside may also have a higher affinity for the external site.

In addition to glucose analogues, a number of structurally unrelated compounds also show asymmetric competitive inhibition of glucose transport. The fungal metabolite cytochalasin B was shown to be a good inhibitor of glucose transport in the erythrocyte by Taverna & Langdon (1973) and Bloch (1973). Jung & Rampal (1975, 1977) reported that cytochalasin B was a competitive inhibitor of D-glucose exchange. When the inhibition by cytochalasin B of infinite cis exit (Sen & Widdas experiment) was studied (Taverna & Langdon, 1973; Bloch, 1973 and Basketter & Widdas, 1977, 1978) the results showed no increase in the

Fig. 2. Possible model for sugar transport in the human erythrocyte



From Barnett et al. (1975)

6-O-propyl-D-glucose (R = C_3H_7; R' = H) can bind to the transport system in conformation A, but is not transported for steric reasons. Similarly, propyl-β-D-glucoside (R = H; R' = C_3H_7) can bind to conformation B but cannot be transported. D-glucose can bind to both conformations and as the transport site changes from conformation A to conformation B is effectively transported from outside tc inside. Only some of the probable hydrogen bonds are shown.

infinite cis Km. These results were interpreted by Basketter & Widdas (1977) as cytochalasin B binding to the internal site only. Basketter & Widdas (1978) also studied the inhibitor phloretin and concluded that phloretin could react with both inner and outer sugar binding sites.

Studies with the irreversible inhibitor 1-fluoro-2-4-dinitrobenzene (FDNB) by Bowyer & Widdas (1958) revealed that inhibition of exit developed more rapidly than inhibition of entry. Further studies by Barnett et al. (1975) revealed that non-transported inhibitors modified at C-4 and C-6 protected against FDNB inactivation whereas those modified at C-1 enhanced FDNB inactivation of the transport system. From this information Barnett et al. proposed that FDNB reacted with the inward facing form of the transporter.

The transport of hexoses in the hepatocyte

Hexoses have been shown to be taken up rapidly by the perfused liver (Williams et al. 1968). The uptake is stereospecific with D-glucose being transported more rapidly than L-glucose. The uptake of D-glucose was inhibited by phloretin.

The use of an isolated hepatocyte preparation (Baur & Heldt, 1977) has allowed kinetic studies of hexose transport to be performed without major assumptions. The isolated hepatocyte preparation is not an homogeneous cell preparation but contains several different types of cell. Baur & Heldt (1977) showed that hexoses were transported into the cell at a rate exceeding that of metabolic conversion. The rate of hexose transport was shown to decrease in the order D-glucose $\sim$ 3-O-methyl-D-glucose > D-galactose > 2-deoxy-D-glucose > D-fructose >> L-glucose. In the presence of metabolic inhibitors, transport of

D-glucose continued until equilibrium was reached, and this result suggested that there was no active accumulation of hexoses. Hexose transport in the hepatocyte was shown to be sodium independent but was inhibited by cytochalasin B, phloretin and phloridzin.

Craik & Elliot (1979) studied the kinetics of 3-O-methyl-D-glucose transport in hepatocytes and showed that 3-O-methyl-D-glucose is transported symmetrically (see Table 1). D-fructose and D-galactose are also transported symmetrically (Craik & Elliot, 1980) but are transported more slowly due to their higher Km value. 3-O-methyl-D-glucose is a competitive inhibitor of D-fructose and D-galactose transport indicating that all these sugars are transported by the same transport system (Craik & Elliot, 1980).

A variety of hormones have been shown to exert effects on hepatic metabolism. Short term changes in glucose production or consumption can usually be correlated with changes in the rate of glycogen degradation or synthesis (Hems & Whitton, 1980). Baur & Heldt (1977) found no effect of insulin on glucose transport in isolated hepatocytes. In the intact perfused liver Mondon et al. (1975) reported a small reduction in D-glucose output in the presence of insulin. This result appears to be due to changes in glycogen metabolism rather than direct effects on the hexose transport system.

Hexose transport in the thymocyte

The thymus is a primary lymphoid organ which supports extensive division of stem cells which go on to form T-lymphocytes. Thymocyte populations can be prepared from the thymus of rats by gentle mechanical disruption (Whitesell et al., 1977). The population of cells produced consists of active cells which equilibrate 3-O-methyl-D-glucose rapidly (minute range) and quiescent cells which equilibrate 3-O-methyl-D-glucose slowly (hours).

Whitesell et al. (1977) studied the kinetics of 3-O-methyl-D-glucose transport in active rat thymocytes and showed that the rate of equilibrium exchange was faster than that of zero trans entry. The presence of 3-O-methyl-D-glucose on the trans face of the membrane (inside the cell) lead to an acceleration of the rate of unidirectional influx. The results of Whitesell et al's experiments are presented in Table 1.

Whitesell & Regen (1978) studied hexose transport in quiescent rat thymocytes and showed the zero trans entry Km to be approximately 25mM and the Vmax to be 0.14μMol/min/ml packed cells. As with active cells quiescent thymocytes showed trans stimulation of the transport rate and also showed accelerated exchange.

Transport in quiescent thymocytes can be stimulated by the addition of a number of compounds such as the calcium ionophore, tetracaine, phenazine methosulphate and arsenate. The stimulation of transport is rapid and does not require protein synthesis (Reves, 1977) indicating that the quiescent cell has a full complement of transport systems. In the thymocyte a stimulation of transport occurs with changes in both the Km and Vmax for transport (Whitesell & Regen, 1978).

Hexose transport in muscle

The hexose transport system of muscle has been shown to have many of the features of a facilitated diffusion process. Norman et al. (1959) Narahara et al. (1960) and Chaudry & Gould (1969) showed saturation kinetics in skeletal muscle. Morgan & Neely (1972) showed the system to be stereospecific and also Morgan et al. (1964) demonstrated counterflow. One of the major difficulties in studying muscle preparations is the preparation of a sample sufficiently thin to allow rapid equilibration of oxygen and substrates between the sample of muscle and the incubation medium. Due to these difficulties detailed kinetic analysis of the transport system has not been performed.

There is no detailed specificity data available for the muscle hexose transport system. D-galactose (Fisher & Lindsay, 1956), D-arabinose, L-arabinose, L-xylose (Carlin & Hechter, 1961), D-xylose (Kipnis & Cori, 1957), D-fructose (Nakada, 1956) and D-glucosamine (Nakada et al., 1955) have all been shown to be transported into muscle by an insulin sensitive route. Since D-glucose is rapidly metabolised by muscle preparations the glucose analogues 3-O-methyl-D-glucose and 2-deoxy-D-glucose have also been used (Morgan et al., 1964 and Narahara and Özand, 1963).

The muscle hexose transport system is regulated by a number of hormones and physiological states. Kohn & Clausen (1971) showed that insulin rapidly stimulates hexose transport in muscle. Ruderman et al. (1971) reported up to 15-fold increases in the rate of hexose transport in muscle with insulin. They also showed that this effect was not coupled to phosphorylation of D-glucose inside the cell. Under basal conditions the concentration of free D-glucose in the cytoplasm is close to zero (Chaudry & Gould, 1969) indicating that the transport of D-

glucose is the rate limiting step in its metabolism.

Stimulation of the hexose transport system in muscle is also brought about by contractile activity (Kohn & Clausen, 1971) though the effect is somewhat smaller and later in onset than that of insulin. Kohn & Clausen also reported that anoxia and metabolic poisons stimulated the rate of hexose transport.

Hexose transport in the small intestine

The epithelial cells of the small intestine actively transport D-glucose from the gut lumen into the bloodstream. Thus D-glucose is transported against its concentration gradient. D-glucose is first transported across the brush border of the epithelial cell by an active transport system coupled to the flux of sodium ions down their concentration gradient. The sodium ion gradient is generated by a separate sodium potassium ATPase. The glucose molecules accumulated in the epithelial cell then diffuse out of the cell by a sodium dependent facilitative diffusion system present in the basal lateral membrane. The sodium dependent and sodium independent hexose transport systems are located at different ends of the cell and can be separated by sub-cellular fractionation of epithelial cells (Murer et al., 1974).

Active hexose transport in the small intestine

Murer & Kinne (1976) studied the transport of D-glucose by brush border vesicles isolated from rat small intestinal epithelium. The transport of D-glucose was increased two-fold when sodium ions were added under non-gradient conditions compared to a similar concentration of potassium ions. In the presence of a sodium isothiocyanate

gradient the rate of D-glucose transport was increased four-fold and there was a transient accumulation of D-glucose within the vesicles. There is also a transsient accumulation of sodium in the vesicles when glucose transport occurs. When a non-penetrating anion such as sulphate was used the rate of glucose transport was considerably reduced. These results are interpreted as electrogenic coupling of the flux of D-glucose to the flux of sodium ions. The rate of transport is then dependent on both the magnitude of the sodium ion gradient and the membrane potential.

A study of the specificity of active transport in the hamster intestine was performed by Wilson & Crane (1958) and Wilson & Landau (1960). The results of these experiments (reviewed by Crane 1960) indicated that a pyranose ring structure with a gluco configuration hydroxyl at C-2 was required for active transport. Crane (1960) also suggested that hexoses in the $^4C_1$ conformation had a higher affinity for the transport system.

Barnett et al. (1968, 1969, 1970) also studied the active transport of hexoses in the hamster small intestine. Their results confirmed those of Crane et al. and in addition they proposed hydrogen bonds to the C-1, C-3, C-4 and C-6 hydroxyls. Barnett et al. also reported that 5-thio-D-glucose had a high affinity for the transporter, and from this observation they concluded that there is no hydrogen bond to the ring oxygen. None of the hydrogen bonds to C-1, C-3, C-4 or C-6 were essential, since analogues in which these hydroxyls were epimerised or removed were still transported, but with reduced affinity. Barnett et al. also studied the transport of halo-substituted sugars. Both chloro and fluoro sugars were transported well, and from this observation Barnett et al. proposed that hydrogen bonds were directed

towards the oxygen of the hydroxyl at C-1, C-3, C-4 and C-6. Chloro or fluoro substitution at C-2 however prevented sodium dependent transport, and Barnett et al. proposed that a covalent bond was formed between the transporter and the C-2 hydroxyl.

The sodium dependent hexose transport system also transports methyl-α-D-glucoside whereas sugars alkylated at C-3 are poorly transported. Muflih & Widdas (1976) showed that 4,6-O-ethylidene-D-glucose was an inhibitor of sodium dependent hexose transport in the rat intestine.

Sodium independent hexose transport in the small intestine

Bihler & Cybulsky (1973) studied the sodium independent hexose transport system by perfusing the gut lumen with a solution of mercuric chloride before preparing isolated cells. This allowed the study of transport in the uninhibited basal lateral membranes. These results together with those of Kimmich & Randles (1975, 1976) showed that sodium independent transport of 2-deoxy-D-glucose and 3-O-methyl-D-glucose occurred via a facilitative diffusion system. The Km for sodium independent D-glucose transport in purified basal lateral membrane vesicles was estimated to be 44mM (Wright et al., 1980). The sodium independent hexose transport system is more sensitive to inhibition by phloretin than phloridzin.

Wright et al. (1980) studied the substrate specificity of the sodium independent hexose transport system in purified basal lateral membranes. They measured the inhibition of D-glucose transport by D-glucose analogues and concluded that there is no requirement for an equatoria hydroxyl except at C-1. Their results showed no requirement for a gluco-configuration C-2 hydroxyl, since D-mannose and 2-deoxy-D-glucose both inhibited D-glucose transport. A study of the spatial requirements

for binding by Wright et al., showed that the sodium independent system is unable to transport methyl-α-D-glucoside unlike the sodium dependent system which transports this analogue. N-acetyl-glucosamine shows a low affinity for the sodium independent transporter indicating the possibility of steric hindrance at C-2. Wright et al. show no evidence for steric hindrance at C-3 and C-4 but 6-O-methylation leads to a reduction in affinity. Wright et al. concluded that the sodium independent hexose transport system of the basal lateral membrane is similar in its substrate specificity to the hexose transport system of the human erythrocyte reported by Barnett et al. (1973a).

Regulation of intestinal transport

Studies on the regulation of active transport of D-glucose in the small intestine are difficult to interpret due to possible changes in intestinal function or metabolism (Crane 1960). Some of the changes observed are due to effects on fast growing tissue, with an individual cell having a useful life span of two or three days. Thus for example the reduction in glucose uptake observed on long term starvation may be related to a loss of cells from the intestine. Crane (1960) observed no effects on hexose transport when insulin was added to intestinal preparations in vitro.

Hexose transport in the kidney

Investigations on renal sugar transport have indicated that the proximal tubule is the main site of sugar reabsorbtion (Walker et al., 1941). Studies by Silverman (1976) and Knight et al. (1977) indicate that several sugar transport pathways with overlapping specificities may be present along the nephron. Kleinzeller et al. (1980) have suggested

that only one of these systems is responsible for the active transport of D-glucose and D-galactose.

The epithelial cells of the proximal tubule actively transport sugars from the interstitial fluid into the cell and then transfer the sugar back into the bloodstream. The mechanism of action of these cells is very similar to that of the epithelial cells of the small intestine (see p.23). As with intestinal cells the active transport of sugars through the brush border is coupled to the flux of sodium ions down their concentration gradient (Crane, 1962). Transport of hexoses across the basal lateral membranes into the bloodstream is by sodium independent facilitated diffusion. Kinne et al. (1975) described the separation of the two activities by means of subcellular fractionation procedures which yield purified brush border and basal lateral membranes.

Sodium dependent sugar transport in the kidney

Kinne et al. (1975) observed a similar sodium dependent accumulation of D-glucose in brush border vesicles isolated from the rat kidney to that observed in brush border vesicles from the intestine.

Silverman et al. (1970) and Silverman (1974) studied renal hexose transport in the whole dog kidney using a multiple indicator dilution technique. In this method material is injected into a tubule and the rate of removal is measured. Their results showed the following sugars to be reabsorbed in order of decreasing affinity. D-glucose > methyl-α-D-glucoside = methyl-β-D-glucoside > D-galactose > 2-deoxy-D-glucose > D-fructose > myoinositol. The affinity of 2-deoxy-D-glucose is low and was estimated to be 2-3 ordersof magnitude less than that of D-glucose. Whilst the methyl α and β D-glucosides were absorbed from the

tubule, reabsorbtion into the blood was negligible.

Since the methyl α and β D-glucopyranoses are only transported by the sodium dependent hexose transport system of the brush border Kleinzeller et al. (1980) studied the inhibition of methyl-β-D-galactoside uptake into rabbit renal cortex using a range of sugar analogues. Their results showed a closed ring structure to be required for sugar transport since 1-deoxy-D-glucose and 1-deoxy-fluoro-D-glucose, which are in a fused ring form are inhibitors of transport. This study also suggested that hydrogen bonds were directed towards C-1, C-2 and C-4 oxygens. There is also a hydrogen bond directed towards the ring oxygen, since 5-thio D-glucose is a poor inhibitor (Whistler & Lake, 1972, Kleinzeller et al., 1980, and Silverman, 1980). The requirement for a C-6 hydroxyl does not seem to be as important as those at other ring positions for binding to the transporter since 6-deoxy-D-glucose inhibited methyl-α-D-glucoside transport. The C-6 hydroxyl is however required for transport as 6-deoxy-D-glucose is not transported. 6-deoxy-6-fluoro-D-glucose did not inhibit methyl-α-D-glucoside transport indicating that this analogue does not interact with the transporter. 2-deoxy-2-fluro-D-glucose is a poor inhibitor of methyl-α-D-glucoside transport in the kidney (as opposed to showing no inhibition in the intestine (Barnett et al. 1970) which suggests hydrogen bonding rather than the formation of a covalent bond to the C-2 hydroxyl. 4,6-O-ethylidene-D-glucose was also shown to interact with the transporter but was not actively transported.

Sodium independent hexose transport in the kidney

The studies of Kinne et al. (1975) showed a stereospecific D-glucose transport system in the basal lateral membranes of rat kidney. This transport was not dependent on the presence of sodium ions and was relatively insensitive to inhibition by phloridzin but strongly inhibited by phloretin. The substrate specificity of this system in the dog kidney has been studied by Silverman et al. (1970) and Silverman (1974, 1980) by studying the removal of material injected via the renal artery. On the basis of their results Silverman et al. proposed that the hydroxyls at C-1 and C-2 and the ring oxygen were important for the binding of D-glucose to the transporter, and they proposed that hydrogen bonds were directed towards these positions.

Hexose transport in the brain

The mammalian brain depends under normal conditions almost exclusively on glucose as its source of energy, and it accounts for about 25% of the total body glucose consumption (Bachelard & McIlwain, 1969). The existence of a selective permeability barrier between blood and brain is well established, and it has been proposed that the capillary endothelial cells limit the movement of solutes into the brain. Glucose transport into the brain through this barrier exhibits the characteristics of a facilitated diffusion system. Buschiazzo et al. (1970) studied 3-O-methyl-D-glucose transport into the whole brain. The transport of 3-O-methyl-D-glucose exhibits saturation kinetics with a Km of approximately 7mM. Buschiazzo et al. also demonstrated counterflow of 3-O-methyl-D-glucose and D-glucose in the whole brain. Glucose transport in the brain is inhibited by phloretin and phloridzin (Betz et al., 1975) and cytochalasin B (Drewes et al., 1977).

Betz et al. (1975 and 1976) have studied the substrate specificity of the D-glucose transporter in whole dog brain by the inhibition of D-glucose uptake by glucose analogues. Their results show competitinn between the analogues, suggesting the presence of a single hexose transport system. Their results showed that a pyranose ring is required for binding to the transporter. There is no absolute requirement for any specific hydroxyl, since epimerisation or removal of a given hydroxyl did not prevent the glucose analogue from binding. The hydroxyl at C-2 is not required since 2-deoxy-D-glucose was transported at a similar rate to D-glucose, yet D-mannose, the C-2 epimer of D-glucose, was a poor inhibitor compared to 2-deoxy-D-glucose, and Betz et al. proposed steric hindrance by a manno configuration hydroxyl. 5-thio-D-glucose, 1-deoxy-D-glucose, i-inositol, D-galactose, D-xylose and L-glucose were all poor inhibitors of glucose uptake by the brain. Betz et al. proposed hydrogen bonds to the ring oxygen C-1, C-3, C-4 and C-6. From these results it was suggested (Betz et al., 1976) that the blood brain barrier hexose transport system has a similar substrate specificity to that of the human erythrocyte (Le Fevre & Marshall, 1958, Barnett et al., 1973a and Kahlenberg & Dolansky, 1972).

Hexose transport has also been studied in isolated brain capillaries (Betz et al., 1979) and synaptosomal preparations (Heaton & Bachelard, 1973). Glucose transport in these fractions is inhibited by Cytochalasin B, phloretin and phloridzin. Phloretin is a more potent inhibitor than phloridzin. There is no effect of ouabain, 2,4,dinitrophenol, or insulin on hexose transport, and hexose transport in these fractions is sodium independent.

Hexose transport in cultured cells

Cultured animal cells have provided a convenient model system for the study of facilitated diffusion. These cells are easily prepared in suspension culture as a homogeneous preparation of isolated cells. Graff et al. (1978) reported that 3-O-methyl-D-glucose was rapidly transported by Novikoff hepatoma cells with a $t_{\frac{1}{2}}$ of between 15 and 40 seconds depending on the substrate concentration. This rapid transport rate requires the use of rapid sampling techniques in order to make accurate determinations of the transport rate (Wohlheuter et al., 1978).

Plagemann et al. (1981) studied the kinetics of 3-O-methyl-D-glucose transport in a range of cultured cell lines. They reported that human HeLa cells, mouse L cells, P388 leukaemia cells, Chinese hamster ovary cells and Novikoff hepatoma cells (Graff et al., 1978) all showed directional symmetry, with the rates of zero trans entry and exit being equal. The rate of equilibrium exchange in these cell lines is however greater than that for zero trans experiments. The human HeLa cell shows K^{ee} = 36mM and V^{ee} = 87.6mM min^{-1} compared to the zero trans experiment where $K^{Zt}_{2-1} = K^{Zt}_{1-2}$ = 20mM and $V^{Zt}_{1-2} = V^{Zt}_{2-1}$ = 33.6mM min^{-1}. There were no significant changes in the kinetics of 3-O-methyl-D-glucose transport in these cell lines when the intracellular ATP levels were depleted by cyanide poisoning and there was no evidence for the active transport of hexoses.

A detailed study of the substrate specificity of these cultured cell lines has not been performed. The study of Plagemann et al. showed that the K^{ee} for D-glucose analogues was in the order 2-deoxy-D-glucose < 3-O-methyl-D-glucose = D-glucose < D-galactose << D-glucosamine. All these sugars are capable of causing trans stimulation of 3-O-methyl-D-glucose and 2-deoxy-D-glucose entry. 2-deoxy-D-glucose and 3-O-methyl-D-

glucose inhibited the transport of each other in a simple competitive manner, and the apparent Ki values were similar to the transport Km values.

Another cultured cell line of great interest is the murine 3T3-pre adipocyte line (Green & Kehinde, 1974). These cells, upon achieving a growth inhibited state, can be transformed into cells possessing many of the biochemical and morphological attributes of adipocytes. The transformation may be brought about by insulin, isobutyl methyl xanthine and dexamethasone (Rosen et al., 1978). After transformation the hexose transport system of these cells is stimulated by the addition of insulin. Rosen et al. (1978) suggested that the stimulation of hexose transport was due to an increase in the Vmax with no effect on the Km which was approximately 2mM for the uptake of 2-deoxy-D-glucose in the presence and absence of insulin.

Other cultured cell lines such as the Novikoff hepatoma cell line can be transformed by stimuli such as mitogens or DNA and RNA tumour viruses so that normal density dependent growth inhibition does not occur. These transformed cells show an increased rate of 2-deoxy-D-glucose uptake (Plagemann & Richey, 1974). Chick embryo fibroblasts have been shown to have an increased hexose transport activity after hexose starvation (Martineau et al., 1972). These effects appear to be independent of phosphorylation and represent increases in the Vmax of the transport system. With the exception of transformed 3T3-pre adipocytes, hormone responses have not been reported in cultured cells.

Hexose transport in the lens of the eye

The lens of the eye offers a convenient tissue in which to study hexose transport. The lens has no vascular system and is dependent on substrate available from the surrounding humours. Lou & Kinoshita (1967) showed that glycolysis is the principal route for metabolism in the lens.

Elbrink & Bihler (1972a) studied the kinetics of 3-O-methyl-D-glucose transport in the lens and showed that 3-O-methyl-D-glucose was transported by a facilitated diffusion process. The Km for 3-O-methyl-D-glucose transport was approximately 90mM. Transport of 3-O-methyl-D-glucose was inhibited by phloretin and phloridzin, with phloretin being the more effective inhibitor. Elbrink & Bihler (1972b) also showed that D-xylose and L-arabinose were transported into the lens but at a lower rate than 3-O-methyl-D-glucose. L-glucose and methyl-α-D-glucoside were not transported. From their results Elbrink & Bihler proposed that the hexose transport system of the lens was similar to that of the erythrocyte.

Free glucose is found in the lens (Lou & Kinoshita, 1967) and transport does not appear to be the rate limiting step in hexose metabolism. Elbrink & Bihler (1972a) found no evidence for hormonal regulation of hexose transport in the eye.

Hexose transport in the placenta

The placenta is primarily an organ for the interchange of material between the fetal and maternal bloodstream without actual mixing of the two bloodstreams. Widdas (1952) proposed that hexoses cross the placental membranes by a facilitated diffusion process. There is no evidence for active transport of hexoses by the placenta since the maternal blood

glucose level is usually equal to or higher than that of the fetal bloodstream. Thus hexoses pass into the fetal circulation by simple diffusion down their concentration gradient. D-fructose was observed to be transported from the maternal to the fetal bloodstream at a much slower rate than D-glucose (Widdas, 1952).

Models for the facilitated diffusion of hexoses

A number of models have been proposed in order to account for the properties of facilitated diffusion systems. Since the hexose transport system of the human erythrocyte is one of the most intensively studied facilitated diffusion systems, the majority of models for sodium independent hexose transport have been developed to account for the asymmetric transport parameters observed in the human erythrocyte.

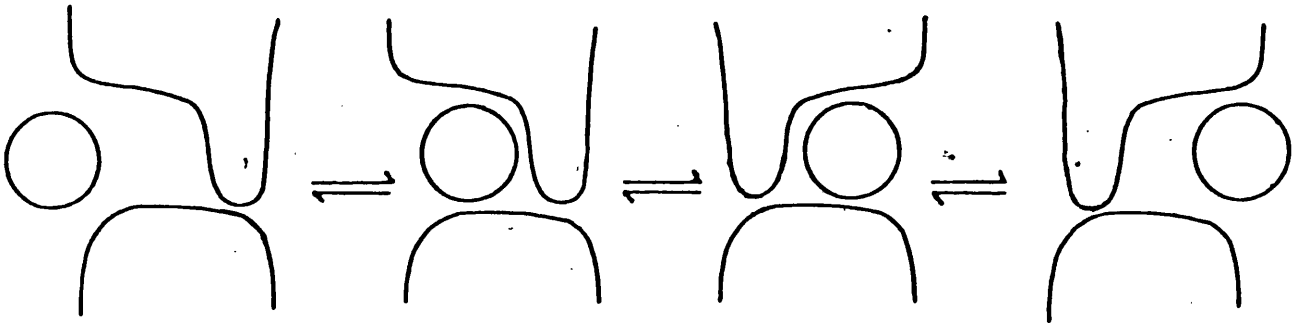
The carrier hypothesis

The lipid soluble carrier model for membrane transport processes was first proposed by Le Fevre (1948) who suggested that the transported molecule could form a complex with a lipid soluble component of the membrane which was able to cross the membrane. This model was further developed by Widdas (1952) who went on to propose that the carrier could return across the membrane in order to ferry another

substrate molecule across. Thus the basic carrier hypothesis calls for a lipid soluble carrier with a specific substrate binding site for the transported substrate. At the cis face of the membrane the substrate binds to the carrier to form a complex which diffuses across the lipid interior of the membrane. At the trans face the complex breaks down to release the substrate into the trans solution. The carrier is then free to return to the cis face of the membrane.

Physical and chemical studies have suggested that this mechanism is unlikely. Singer & Nicolson (1972) suggested that the rate of diffusive or rotational movement of membrane proteins across the membrane is slow compared to the rate which would be required for a facilitated diffusion system. Also, studies on other transport systems have suggested that the movement of a protein molecule within the bi-layer is unnecessary for translocation to occur. Kyte (1974, 1975) reported that the protein forming the sodium potassium ATPase is exposed on both faces of the membrane simultaneously and that there is no reorientation of the molecule during transport. Similar observations have been made on the sarcoplasmic calcium magnesium ATPase (Martonosi & Fortier, 1974 and Dutton et al., 1976).

Vidaver (1966) suggested that the mobile component of the carrier system may be as small as the movement of one or two hydrogen bonds. Thus a conformational change in a trans membrane protein would lead to the conversion of an outward facing site to an inward facing site bringing about the transfer of substrate across the membrane. Such a system would be consistent with the studies of Barnett et al. (1973b, 1975) who showed different spatial requirements for the inside and outside sites. A scheme for this carrier model can be drawn as



The kinetic predictions of this model will be identical to those of the lipid soluble carrier and will be discussed later. Thus the different rates of diffusion for loaded and unloaded lipid soluble carrier can be interpreted as different rates of conformational change for the loaded and unloaded forms of the transmembrane protein.

Pore models

The simplest of all models for facilitated diffusion is the simple pore model. In this model a water filled pore spans the membrane allowing a continuous passage for solute through the membrane (Danielli, 1954). The presence of a limited number of pores will give rise to saturation kinetics (Stein & Danielli, 1954, and Zierler, 1961). In order that transport through pores is stereospecific at least one recognition site is required, with binding to this site being required before translocation can occur (Adair, 1956). Some form of restriction in the pore is also required in order to prevent molecules smaller than the substrate passing freely through the membrane.

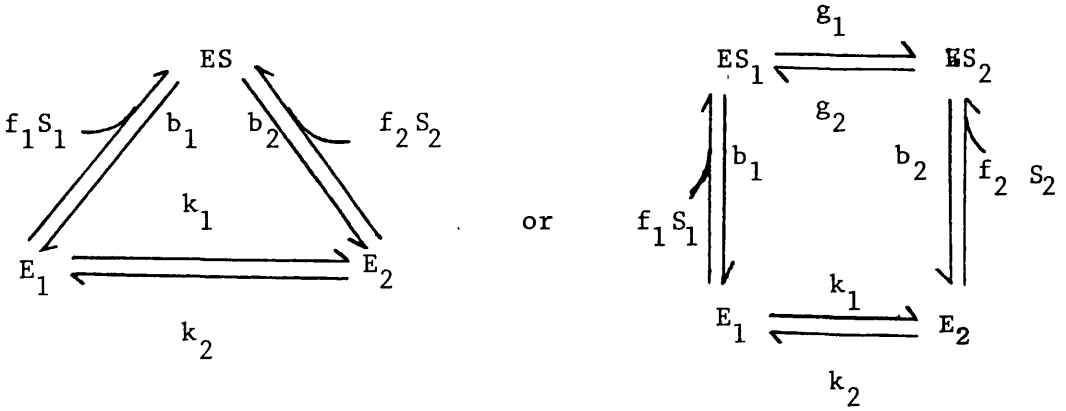
Physical studies are consistent with the model of a water filled route through the membrane. Naftalin & Holman (1977) showed sugar dependent increases in the water content of human erythrocyte membranes. This increase was prevented by the addition of mercuric chloride which inhibits transport activity. Bowman & Levitt (1977)

have studied the glucose dependent permeability of a series of 4 to 6 carbon polyols in erythrocyte membranes. Their results indicate that polyol permeability is inversely proportional to molecular size and they suggest that this may be related to the ability of these compounds to enter a pore. The volume of a typical membrane protein spanning the membrane has been estimated at at least 2.5×10^5 $\mathring{A}^3$ (Holman, 1980) which gives a space within the molecule capable of allowing two sugar molecules (of 1.25×10^2 $\mathring{A}^3$ each) to pass one another.

Kinetic predictions of models

Carrier models

The kinetics of carrier mediated transfer have been developed by a number of authors (Widdas 1952, Regen & Morgan, 1964, and Stein & Lieb, 1972). A reaction scheme can be drawn for carrier models in which a single substrate molecule (S) combines with the carrier (E). The subscripts 1 and 2 refer to the two faces of the membrane.



Using steady state kinetics these two reaction schemes are indistinguishable (Lieb & Stein, 1972). Pre-steady state kinetic analysis is required to identify different forms of the carrier substrate complex. Lieb & Stein (1972, 1974a) have derived a steady state equation

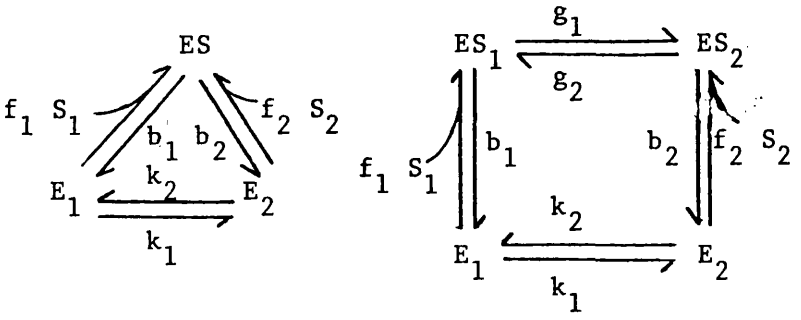
for the unidirectional flux of substrate through this model.

$$U_{1\to2} = \frac{K_{12}\, S_1 + S_1\, S_2}{K_{12}\, K_{21}\, Roo + K_{21}\, R_{12}\, S_1 + K_{12}\, R_{21}\, S_2 + Ree\, S_1\, S_2} \qquad (2)$$

The unidirectional flux equation for flux in the reverse direction ($U_{2\to1}$) is given by reversing the subscripts in equation (2). The parameters for this equation are defined in Table 2 and describe the movement of the different forms of the carrier across the membrane in different directions when loaded with substrate or unloaded. Terms in R are sums of specific resistances for the movement of the carrier in its different forms. Thus R_{12} is the sum of the specific resistance of loaded carrier moving in the direction 1 to 2 and the specific resistance of the unloaded carrier returning in the direction 2 to 1. Ree is the sum of the specific resistance of loaded carrier moving in the direction 1 to 2 and the loaded carrier moving in the direction 2 to 1. Roo is similarly the sum of resistances to movement of the unloaded carrier in both directions. K is the operative affinity of the system for substrate when the system is unperturbed by the presence of substrate.

For a symmetrical carrier the sites on faces 1 and 2 have equal affinities for substrate i.e. $f_1 = f_2$ and $b_1 = b_2$. Regen & Morgan (1964) showed that unequal rates of movement of loaded and unloaded carrier within the membrane would lead to asymmetry. Thus for the erythrocyte Ree < Roo. Lieb & Stein (1972) showed that this assumption predicts that the Km for the infinite cis experiment should be greater than or equal to one half of the Km for equilibrium exchange. This observation provides an important rejection criterion for this model.

Table 2. Steady state solutions for the one and two complex forms of the simple carrier



	one complex model	two complex model
$n\,R_{12}$	$\frac{1}{b_2} + \frac{1}{k_2}$	$\frac{1}{b_2} + \frac{1}{k_2} + \frac{1}{g_1}\left(\frac{b_2 + g_2}{b_2}\right)$
$n\,R_{21}$	$\frac{1}{b_1} + \frac{1}{k_1}$	$\frac{1}{b_1} + \frac{1}{k_1} + \frac{1}{g_2}\left(\frac{b_1 + g_1}{b_1}\right)$
$n\,R_{oo}$	$\frac{1}{k_1} + \frac{1}{k_2}$	$\frac{1}{k_1} + \frac{1}{k_2}$
$n\,R_{ee}$	$\frac{1}{b_1} + \frac{1}{b_2}$	$\frac{1}{b_1} + \frac{1}{b_2} + \frac{1}{g_1}\left(\frac{b_2 + g_2}{b_2}\right) + \frac{1}{g_2}\left(\frac{b_1 + g_1}{b_1}\right)$
K_{12}	$\frac{k_1}{f_1} + \frac{k_2}{f_2}$	$\frac{k_1}{f_1} + \frac{k_2}{f_2}\left(\frac{b_2 + g_2}{g_2}\right)$
K_{21}	$\frac{k_2}{f_2} + \frac{k_1}{f_1}$	$\frac{k_2}{f_2} + \frac{k_1}{f_1}\left(\frac{b_1 + g_1}{g_1}\right)$

From the law of microscopic reversibility $b_1 k_1 f_2 = b_2 k_2 f_1$ and $g_1 b_2 k_2 f_1 = g_2 b_1 k_1 f_2$

then $R_{ee} + R_{oo} = R_{12} + R_{21}$

also $K_{12} = K_{21}$

Where n is the total number of carriers per unit membrane.

Geck (1971) proposed an asymmetric carrier model with unequal half saturation constants on either side of the membrane. The unidirectional flux through this model is also given by equation 2. Since a facilitated diffusion system must achieve equilibrium with no accumulation on one side of the membrane, the Haldane relationships for net entry and exit must be the same i.e.

$$\frac{Vi}{Ki} = \frac{Vo}{Ko}$$

where i and o refer to the inside and outside respectively. Thus the presence of asymmetric affinities implies there must also be asymmetric Vmax values.

The values of the basic carrier parameters can be determined experimentally since equation 2 reduces to a Michaelis Menten form for all the experimental protocols described earlier (Lieb & Stein, 1972). The Km and Vmax values given by different experimental protocols are related to the different parameters, and these relationships are given in Table 3. To test the asymmetric carrier model the values for the model parameters are estimated using the complete range of protocols. If the asymmetric carrier model holds the values for the different parameters should be identical regardless of the protocol used to estimate them.

Regen & Tarpley (1974) proposed an asymmetric carrier model with the additional assumption of unstirred layer effects. This model assumes a relatively thick unstirred layer effect at the inner membrane surface and accounts for most of the observed operational transport parameters in the erythrocyte. It cannot, however, account for the low Km for infinite cis entry of D-glucose in the erythrocyte since the affinity

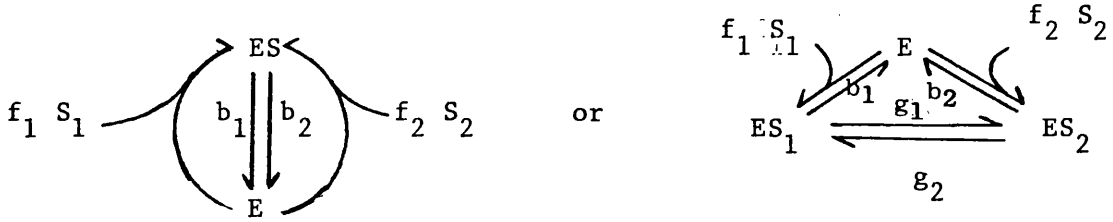
Table 3. The evaluation of the kinetic parameters of the carrier model

procedure	Vmax	Km
Zero trans	$V_{12}^{Zt} = \frac{1}{R_{12}}$	$K_{12}^{Zt} = K_{12}\frac{R_{oo}}{R_{12}}$
	$V_{21}^{Zt} = \frac{1}{R_{21}}$	$K_{21}^{Zt} = K_{21}\frac{R_{oo}}{R_{21}}$
Infinite cis	$V_{12}^{ic} = \frac{1}{R_{12}}$	$K_{12}^{ic} = K_{21}\frac{R_{12}}{R_{ee}}$
	$V_{21}^{ic} = \frac{1}{R_{21}}$	$K_{21}^{ic} = K_{12}\frac{R_{21}}{R_{ee}}$
Equilibrium exchange	$V^{ee} = V_{12}^{ee} = V_{21}^{ee} = \frac{1}{R_{ee}}$	$K_{12}^{ee} = K_{12}\frac{R_{oo}}{R_{ee}}$
		$K_{21}^{ee} = K_{21}\frac{R_{oo}}{R_{ee}}$
		$K_{12}^{it} = K_{12}\frac{R_{21}}{R_{ee}}$
Infinite trans	$V^{it} = V_{12}^{it} = V_{21}^{it} = \frac{1}{R_{ee}}$	
		$K_{21}^{it} = K_{21}\frac{R_{12}}{R_{ee}}$

of the inside site must be low in all circumstances.

Pore models

The simplest of pore models is that of a narrow pore which can allow a single substrate molecule to pass through the membrane. Substrate (S) and pore (E) combine to form a complex whilst the substrate crosses the membrane from side 1 to side 2. A kinetic scheme for this transfer can be drawn.



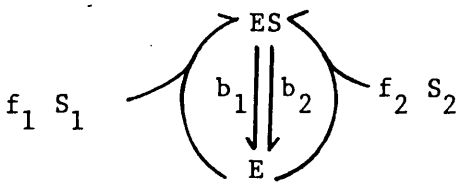
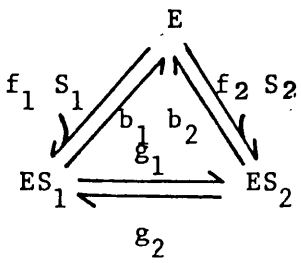
Lieb & Stein (1974b) have derived a unidirectional flux equation for this model. As with the carrier model steady state analysis cannot reveal different forms of the complex and therefore the same equation holds for both schemes.

$$U_{1\to2} = \frac{Q_{21}\ S_1}{Q_{12}\ Q_{21} + Q_{21}\ R_{12}\ S_1 + Q_{12}\ R_{21}\ S_2} \qquad (3)$$

These parameters are defined in Table 4. As with the carrier model the unidirectional flux in the reverse direction is given by reversing the subscripts. The values for the pore parameters can be determined experimentally since equation 3 reduces to a Michaelis Menten form for zero trans and equilibrium exchange experiments (Table 5).

The major prediction of this model is that trans inhibition of unidirectional flux will occur (Hodgkin & Keynes, 1956) so that as S_2 approaches infinite concentration $U_{1\to2}$ will approach zero.

Table 4. Kinetic parameters for the simple pore

	one complex model	two complex model
		
n R_{12}	$\frac{1}{b_2}$	$\frac{1}{b_2} + \frac{1}{g_1}\left(\frac{b_2 + g_2}{b_2}\right)$
n R_{21}	$\frac{1}{b_1}$	$\frac{1}{b_1} + \frac{1}{g_2}\left(\frac{b_1 + g_1}{b_1}\right)$
n Q_{12}	$\frac{b_1}{f_1}\left(\frac{1}{b_1} + \frac{1}{b_2}\right)$	$\frac{b_1}{f_1}\left[\frac{1}{b_1} + \frac{1}{g_1}\left(\frac{b_2 + g_2}{b_2}\right)\right]$
n Q_{21}	$\frac{b_2}{f_2}\left(\frac{1}{b_1} + \frac{1}{b_2}\right)$	$\frac{b_2}{f_2}\left[\frac{1}{b_2} + \frac{1}{g_2}\left(\frac{b_1 + g_1}{b_2}\right)\right]$

where n is the total number of pores per unit membrane.

From the law of microscopic reversibility $b_1 f_2 = b_2 f_1$ and $b_1 f_2 g_2 = b_2 f_1 g_1$. Hence $Q_{12} = Q_{21}$.

Table 5. Experimental determination of parameters for simple pore

procedure	Vmax	Km
zero trans	$V^{Zt}_{1-2} = \frac{1}{R_{12}}$	$K^{Zt}_{1-2} = \frac{Q_{12}}{R_{12}}$
	$V^{Zt}_{2-1} = \frac{1}{R_{21}}$	$K^{Zt}_{2-1} = \frac{Q_{21}}{R_{21}}$
equilibrium exchange	$V^{ee} = V^{ee}_{1-2} = V^{ee}_{2-1} = \frac{1}{R_{12} + R_{21}}$	$K^{ee}_{1} = \frac{Q_{12}}{R_{12} + R_{21}}$

Bypass pore models

More complex pore models have also been proposed in which the pore can be occupied by several substrate molecules at one time. In these models substrate molecules can pass one another within the pore, and therefore trans inhibition does not occur.

The polar creep model

Bowyer & Widdas (1956) proposed a model in which hexose molecules pass down a series of hexose binding sites in the pore. As considered previously, steady state analysis cannot differentiate between the different intermediates; however, for this type of model the half saturation constants for each face of the membrane can be determined (Widdas, 1980). The half saturation constants for the two faces are given by $\emptyset$ and $a\emptyset$ respectively where a is the asymmetry of affinities, thus for a symmetric model a = 1. For net transfer from side 1 to side 2 to occur the substrate concentration at side 2 must be less than that at side 1. Thus the probability of net transfer from side 1 to side 2 is given by a rate constant (V_1) multiplied by the probability of saturation of side 1 times the probability that the site on side 2 is empty.

$$\text{Transfer}_{1\rightarrow 2} = V_1 \frac{c_1}{c_1 + \emptyset} \cdot \frac{a\emptyset}{c_2 + a\emptyset} \qquad (4)$$

where c_1 and c_2 are the substrate concentrations at sides 1 and 2 respectively. A similar equation holds for transfer in the reverse direction except that the rate constant will be V_2.

$$\text{Transfer}_{2\to1} = V_2 \frac{c_2}{c_2 + a\phi} \cdot \frac{\phi}{c_1 + \phi}$$

In order that this model can achieve true equilibrium and does not lead to accumulation of substrate on one side of the membrane $V_2 = aV_1$. When both sites are saturated an exchange of substrate molecules occurs between the two sites. The rate constant for this exchange is Vex and, the unidirectional flux will be given by

$$U_{1\to2} = \frac{V_1 c_1 \cdot a\phi + Vex\ c_1 c_2}{(c_1 + \phi)(c_2 + a\phi)}$$

When a = 1 this model predicts symmetrical transport parameters. Also the Vmax for exchange may be greater than the zero trans Vmax. When $a \neq 1$ the Km and Vmax for zero trans experiments will be asymmetric. The infinite cis experiment Km's will also be asymmetric for this model. Thus the model gives similar predictions to the asymmetric carrier.

Lattice pores

The lattice pore model of Naftalin (1970) is similar to the polar creep model, in that hexose molecules pass through the membrane by one dimensional diffusion between binding sites. This model was initially developed to account for the heteroexchange results of Miller (1968) who observed that the exit of glucose was accelerated more by galactose or mannose than by glucose in the trans solution. Thus Naftalin proposed that the exchange of two different substrates between adjacent binding sites has a higher probability of occurring than simple migration of a single substrate molecule to an adjacent site. Similarly the exchange of two different substrates between a surface binding site and the bulk solution is more likely than dissociation of the substrate from the pore. Computer simulations of

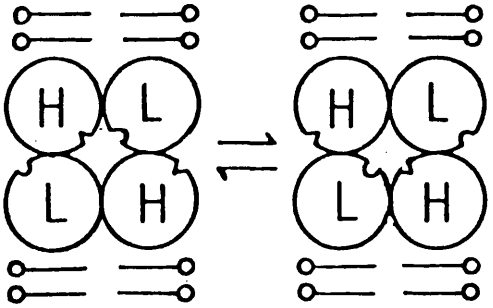
this model (Naftalin,1970) predict accelerated heteroexchange and uphill counterflow. In order to fully simulate Miller's (1968) results an unstirred layer effect was proposed by Naftalin. This model is however symmetrical with respect to the rates of zero trans entry and exit.

Le Fevre's introverting hemiport model

Le Fevre (1973) proposed a model in which microcarriers in the membrane acted as gates to an intramembrane pool. These microcarriers were proposed to be able to face either inwards towards the intramembrane pool, or outwards to contact the solution. Le Fevre proposed that the probability of the microcarriers facing inward or outward was altered by the binding of substrate. Computer simulations based on a change in the probability of the site facing inward of 0.3 for unloaded to 0.99 when loaded were performed by Le Fevre (1973). These computer simulations predicted a high Km for zero trans exit and a low Km for both infinite cis entry and exit. The Km and Vmax for equilibrium exchange were both high. The model is however symmetrical with respect to the zero trans Km's.

The tetramer model

Lieb & Stein (1970, 1971) proposed the tetramer model in which two pairs of subunits at each face of the membrane act as microcarriers to a pore. The two pairs of subunits have different affinities for the substrate; one pair has low affinity (L site) whilst the other pair has high affinity (H site). A diagrammatic representation of the model can be shown as



At low substrate concentrations the substrate molecule being transported remains preferentially bound to the high affinity site. The interconversion of subunits from an outside to an inside facing form is substrate induced. The model predicts the presence of two operational affinities on each face of the membrane. The zero trans Km is determined primarily by the low affinity site (the L site) whereas the infinite cis Km is determined by the high affinity site. This model is symmetrical with the rates of net entry and exit being equal.

The pore of Foley et al.

Foley et al. (1980a) proposed a model where a water filled pore spans the membrane. The pore is gated at each end by a microcarrier. The kinetics of this model are complex with 11 different parameters describing the flux of substrate through the pore. A full kinetic analysis of this model has not been carried out though the model predicts that the Vmax for net flux through the pore will be governed by the rate limiting microcarrier (Holman et al., 1981). Such a model may therefore show similar kinetics to the model of Regen & Tarpley (1974) of an asymmetric carrier with an unstirred layer.

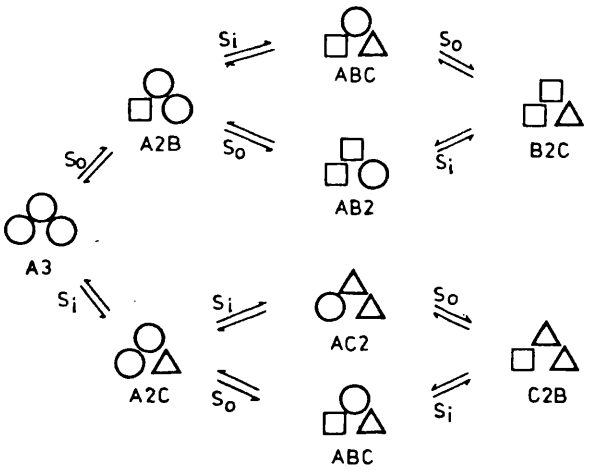
The allosteric pore

Holman (1980) described a model for hexose transport with a protein lined pore composed of three subunits. The points of contact between these subunits form the gates to the pore. Substrate molecules are able to bind to each subunit. The subunits are proposed to be capable of adopting three conformations: conformation A being unoccupied, conformation B is adopted when a substrate molecule is bound from the outside and conformation C is adopted when substrate is bound from the inside. The pore can be occupied by a maximum of three substrate molecules at any given time and all the possible subunit conformations are shown in Fig. 3.

Monovalent occupancy of the allosteric pore by a substrate molecule leads to low Km and Vmax transport parameters since monovalent occupancy does not lead to destabilisation of the gate trans to the occupied subunit. Monovalent occupancy also leads to the exposure of a high Km site (negative cooperativity) on a neighbouring subunit. If the substrate concentration is sufficiently high, then binding to this site leads to the destabilisation of the trans gate leading to a high Vmax for transport. Thus monovalently occupied pores (A2B and A2C forms) show low Km low Vmax parameters for transport whilst divalently occupied pores (AB2 and ABC forms) show high Km high Vmax transport parameters. Equilibrium exchange occurs in a similar manner with low Km and Vmax exchange in the ABC configuration and high Km and Vmax exchange occurring in B2C and C2B forms of the pore.

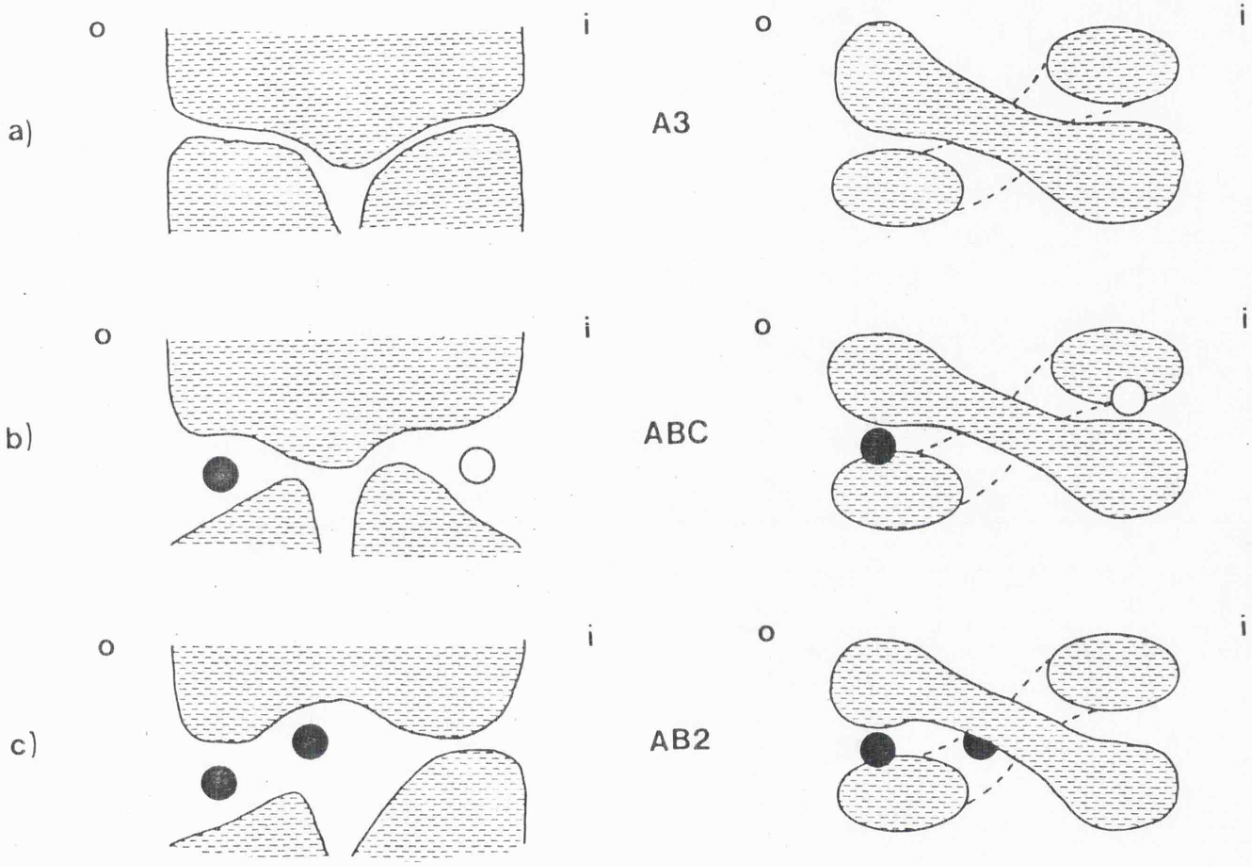
The unidirectional flux equation for zero trans entry through the allosteric pore was derived by Holman et al. (1981) and is:

Fig. 3a. The allosteric pore



A schematic representation of the allosteric pore showing all the proposed occupancy states. (O) A subunits, (□) B subunits, (△) C subunits. A linear arangement of subunits is assumed.

Fig. 3b. The allosteric pore



A schematic representation of the subunit interactions occurring in the allosteric pore.

a) In the absence of substrate the interfaces of the subunits are stable and the pore is closed.

b) The ABC form of the pore where the pore is occupied from the outside and inside by substrate.

c) When the pore is doubly occupied from the outside the subunit interfaces (pore gates) are greatly destabilised and negative cooperativity results. The right panel of this scheme shows cross sections through the pore. The dotted line indicates that two subunits with separate domains at inner and outer surfaces would be equivalent to a linear arrangement of three subunits since both arrangements have inner and outer subunit interfaces.

$$u_{oi} = \frac{Tot\ (V_L\ K_S\ S_o + V_H\ K_S^2\ K_{BB}\ S_o^2}{1 + K_S\ S_o + K_S^2\ K_{BB}\ S_o^2} \qquad (6)$$

where Tot is the total concentration of all forms of the transporter, S_o is the outside substrate concentration, K_S is the subunit association constant, K_{BB} is the subunit interface stability constant for a BB interface and V_L and V_H are the Vmax values for the singly and doubly occupied forms of the pore. The flux equation for zero trans exit is similar except that the subunit interface stability constant is K_{CC}. If the subunit interface stability constants are unequal then the allosteric pore will show asymmetric rates of zero trans entry and exit. Holman et al. (1981) also derived a unidirectional flux equation for equilibrium exchange.

$$u_{oi} = \frac{Tot\ S\ (vK_{LE} + vK_{HE}\ S)}{1 + S\ K_{LE} + S^2\ K_{HE}} \qquad (7)$$

where $vK_{LE} = V_L\ K_S$

$$vK_{HE} = V_{HE}\ K_S^2\ (K_{BB} + K_{CC})\ K_{BC}$$

$$K_{HE} = K_S^2\ (K_{BB} + K_{CC})\ K_{BC}$$

K_{BC}, K_{BB} and K_{BC} are subunit interface stability constants for BC, BB and CC interfaces. V_{HE} is the high Vmax for exchange in the triply occupied allosteric pore.

Due to the complex kinetics of this model detailed predictions of the model depend upon the choice of values for the seven independent parameters. Holman (1980) estimated these parameters for the erythrocyte

hexose transporter using computer simulations. The model shows slight evidence for nonlinearity of reciprocal plots under some conditions (due to the negative cooperativity). For the estimated values for the parameters for human erythrocyte hexose transport, the combinations of low K and low V, and high K high V transport parameters, for the transport of a single substrate reduced the nonlinearity to barely detectable levels. If $K_{BB} > K_{CC}$ then the model shows asymmetric rates for zero trans experiments with a higher Km and Vmax for efflux than for influx. The model also shows accelerated exchange and low symmetrical Km's for infinite cis and infinite trans experiments.

The regulation of carbohydrate metabolism

Circulating blood glucose is derived from two major sources; dietary carbohydrates and the process of gluconeogenesis. An intricate control system is responsible for the maintenance of blood glucose levels at between 60-110mg/100ml of blood in normal men. Blood glucose is the major energy source for the central nervous system, red blood cells, the adrenal medulla and bone marrow. Undoubtedly, the fine control of glucose levels has evolved to serve these functions.

Most ingested carbohydrate is converted to glucose and the resulting post absorbtive raised blood glucose level stimulates the release of insulin from the β islets of the pancreas. This process is mediated by mechanisms involving glucose, glucagon, and vagus impulses. In conditions of lowered blood glucose following starvation, blood glucose levels are replenished by glycogenolysis (via catecholamines and glucagon), gluconeogenesis (via cortisol), lipolysis (via growth hormone), decreased glucose utilisation, and increased fatty acid oxidation.

The major target tissues for hormones involved in glucose homeostasis are muscle, adipose tissue and the liver. The liver is mainly concerned with glucose homeostasis through the formation of glycogen or the formation of carbohydrates from non carbohydrate sources. As discussed previously (p. 20) there is no evidence that hepatic glucose metabolism is regulated by changes in hexose transport across the hepatocyte membrane. In contrast hexose metabolism in both muscle (see p. 22) and adipose tissue (see p. 10) is rate limited by the transport of hexoses in these tissues. In both muscle and adipose tissue the hexose transport system is subject to hormonal regulation.

The regulation of hexose transport

The regulation of hexose transport offers a convenient site for the regulation of hexose metabolism. Due to the technical difficulties of studying hexose transport in muscle the isolated adipocyte has provided a convenient model system in which to study the regulation of hexose transport. A large number of compounds of both physiological and non-physiological relevance have been shown to alter the rate of sugar transport. Unfortunately the lack of a relationship between the effects of these compounds has reduced their usefulness in providing an insight into the mechanism of the regulation of transport.

Hormonal regulation of hexose transport

Insulin

Insulin is a polypeptide hormone of approximately 6500 daltons molecular weight. The major effect of insulin on its target tissues is to stimulate the uptake of sugars, amino acids, fatty acids, ions, and nucleic acid precursors (Pilkis & Park 1974). To date however it has not been possible to ascribe all of insulin's effects on target tissues to changes in membrane transport.

The initial step in the action of insulin is now recognised as binding to receptors in the plasma membrane (Czech 1977). It has also been demonstrated that insulin is able to enter the cell and bind to receptor sites within the cell (Goldfine 1977). The interaction of insulin with its plasma membrane receptors shows reversible non-covalent binding with negative cooperativity (Kahn 1975). Insulin receptors are also present in a number of non-target tissues and the binding of insulin has been described in many tissues, including adipocytes, muscle, liver, lymphocytes, erythrocytes, placenta and fibroblasts. (for recent review see Goldfine 1981). In addition to the plasma membrane, insulin binding

sites have been described in nuclei (and nuclear membranes), smooth and rough endoplasmic reticulum and golgi apparatus (Goldfine 1981).

Cuatrecasas (1969) first coupled insulin to sepharose beads and reported that this conjugate showed full biological activity. Since the sepharose bead was much larger than the cell it was suggested that insulin did not need to enter the cell. Garwin & Gelehrter (1974) showed that free insulin was able to leak out of such conjugates and, the free insulin had biological activity. Insulin has since been coupled to agarose via biotin avidin reactions (May et al., 1978). This derivative has reduced biological activity although even these complexes may still be able to release sufficient free insulin to cause biological activity.

Goldfine et al. (1978) studied the fate of radioactive insulin in mouse fibroblasts following its binding to cell surface receptors. In a similar study Schlessinger et al. (1978) used an insulin molecule which was covalently linked to a fluorescent dye to study the distribution of insulin in cultured IM9 lymphocytes. The results of both experiments showed insulin to bind diffusely over the whole cell surface and then, within a few minutes, the hormone receptor complexes aggregated into patches on the cell surface. These patches were then endocytosed to a location on the rough and smooth endoplasmic reticulum. The incubation of cells with insulin leads to a time and dose dependent decrease in the number of cell surface insulin receptors (Neville & Chang, 1978) with such effects being observed within 10 minutes (Goldfine, 1981).

The multiple effects of insulin on target cells can be separated into three classes:-

Rapid (occurring in seconds) - these are principally effects on the plasma membrane and constitute a change in the rate of transport and a change in the membrane potential.

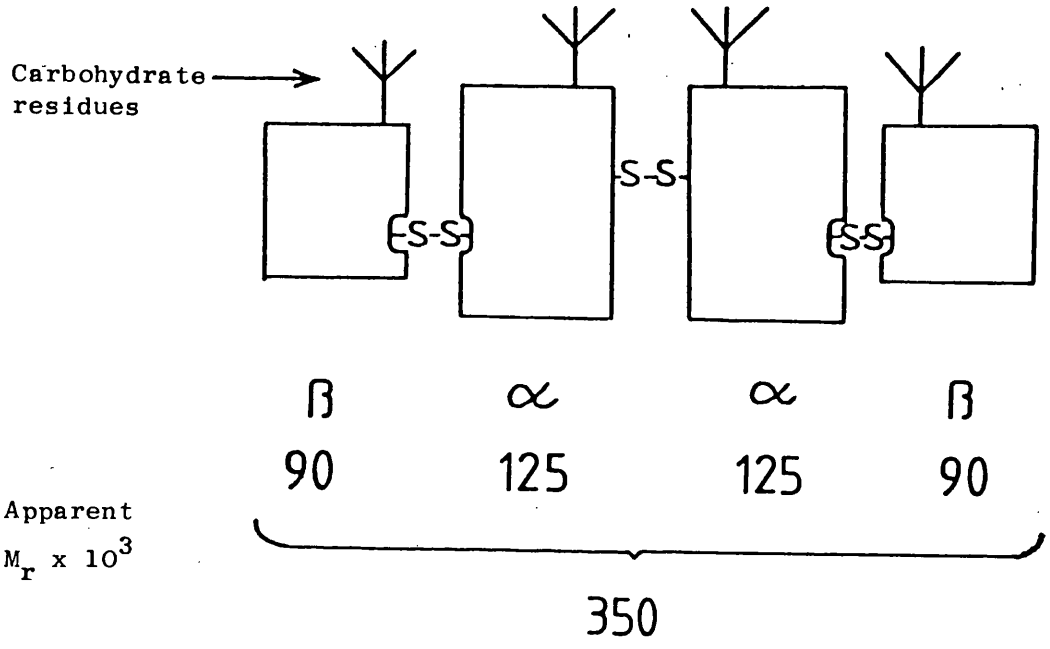
Intermediate (occurring in minutes) - these effects occur at a number of sites within the cell. Cytosolic, mitochondrial and endoplasmic reticulum enzymes are activated, and there is increased protein synthesis at the ribosomal level. There is also an inhibition of protein degradation in lysosomes.

Delayed (occurring in hours) - these are principally effects at the nucleus on the modulation of DNA and RNA synthesis.

In view of these multiple effects of insulin on target cells it is possible that the actions of insulin could be mediated by binding to both surface and intracellular binding sites.

The insulin receptor

Detailed studies on the insulin receptor from a number of tissues indicate that it is an intrinsic membrane glycoprotein of apparent molecular weight 350,000 daltons (Czech et al., 1981). Electrophoretic studies by Massague et al. (1980) indicate that it is composed of two types of subunit, α and β of apparent molecular weights, 125-130,000 daltons and 90,000 daltons respectively. In the plasma membrane the receptor exists in $\alpha\beta$ and $(\alpha\beta)_2$ complexes with the subunits linked by disulphide bonds (Massague & Czech, 1980). The $\alpha\beta$ complex is able to bind insulin, though the subunits do not appear to become physically dissociated when the disulphide bridges linking them are reduced. The subunit composition of the insulin receptor can be expressed diagrammatically.



In order to elicit a maximal biological response from the target cell insulin needs to bind to between 2 and 5% of the total receptors present (Kono & Barham, 1971). Within 30 to 90 seconds of this binding a large increase in the rate of 3-O-methyl-D-glucose transport is observed (Kono et al., 1976, and Harring et al., 1978). Kinetic analysis of the parameters for 3-O-methyl-D-glucose transport in basal and insulin stimulated cells (Whitesell & Gliemann, 1979, Czech, 1976[a], Vinten, 1978, Vinten et al., 1976, and Taylor & Holman, 1981) have shown that the increased rate is due to an increase in the Vmax for transport with no apparent change in the Km. The values of the kinetic constants measured by the different investigators showed some variation; this appears to be

due to different times being used for the measurement of initial rates. Too long a time point has been shown by Taylor & Holman (1981) to lead mainly to an underestimate of Vmax. Czech (1980) has also proposed that the variation may be due to different collagenase preparations being used by different authors.

The activation of hexose transport by insulin was shown to require ATP by Gliemann et al., 1975 and Kono et al.,(1976) who showed that the addition of uncouplers of oxidative phosphorylation (which reduce cellular ATP) prevented the stimulation of adipocytes by insulin. A similar energy requirement is also shown for the inactivation of hexose transport following the removal of insulin (Vega et al, 1980, Laursen et al., 1981). Kono et al. (1977) also showed in a similar study that insulin was not internalised in the presence of metabolic uncouplers. However no effect of ATP depletion was observed on the binding of insulin to the membrane of isolated fat cells (Kono et al., 1977). The use of protein synthesis inhibitors such as cyclohexamide (Fain, 1964) have shown that the activation of hexose transport by insulin does not require de novo synthesis of protein.

Studies on the transport of D-glucose in plasma membranes isolated from rat adipocytes (Martin & Carter (1970); Carter et al. (1972); Chandramouli et al. (1977) and Ludvigsen & Jarett (1979, 1980)) have shown that plasma membranes isolated from basal adipocytes did not show an increase in the rate of D-glucose transport on the addition of insulin, even in the presence of ATP. Plasma membranes which were isolated from cells which had been stimulated with insulin exhibited an increased rate of D-glucose transport (Carter & Martin, 1970). This fossil effect of insulin is remarkably stable and persists even after

the extraction of extrinsic membrane proteins (Pillion et al, 1978). These studies showed that in addition to ATP an intact cell structure is also required for insulin to stimulate hexose transport.

Catecholamines

Catecholamines (adrenaline and noradrenaline) are known to reverse the effects of insulin on lipogenesis and also bring about breakdown of glycogen. Adrenaline stimulation of isolated adipocytes has been shown to increase the rate of glucose oxidation (Cahill et al., 1960, and Luzio et al., 1974). Ludvigsen et al. (1980) studied the uptake of 3-O-methyl-D-glucose by isolated adipocytes and showed a two-fold increase in the rate of glucose transport when 0.25μM adrenaline was present. Ludvigsen et al. also showed a fossil effect of adrenaline in plasma membranes similar to that of insulin. Kinetic analysis of this effect revealed that the increased rate of transport was due to an increase in the Vmax for transport rather than a change in the Km.

The regulation of glycogen metabolism by adrenaline has been shown to involve increase in the intracellular concentration of its second messenger cyclic AMP (Robinson et al., 1971). A number of compounds which mimic adrenaline's action through binding to surface adrenergic receptors have been described. The use of these compounds has revealed two classes of adrenaline receptors in the plasma membrane. These receptors have been designated as α and β adrenergic receptors (Stein & Hales, 1972). It is generally regarded that the activation of lipolysis and the activation of adenyl cyclase are mediated via β receptors, which are part of the adenyl cyclase complex in the plasma membrane. Luzio et al. (1974) showed that the adrenaline stimulation of glucose oxidation could continue in the presence of propranalol

(a β adrenergic blocker) at concentrations which prevented increases in the intracellular levels of cyclic AMP and the stimulation of lipolysis. Conversely the α adrenergic blocking agent phenoxybenzamine prevented the stimulation of glucose oxidation by adrenaline at concentrations which did not inhibit lipolysis and cyclic AMP formation. The presence of phenoxybenzamine had no effect on the stimulation of glucose oxidation by insulin. Luzio et al. concluded that a substantial portion of the adrenaline stimulation of glucose oxidation in rat adipocytes is not mediated by cyclic AMP.

In contrast the results of Ludvigsen et al. (1980) indicated that a large part of adrenaline's action on hexose transport may be mediated by β adrenergic receptors, as in their experiments the stimulation of glucose transport was blocked by propranalol. Phenylephrine an α directed adrenaline analogue produced only slight stimulation of hexose transport.

Thus the mechanism of adrenaline stimulation of hexose transport is unclear and the role of cyclic AMP cannot be eliminated.

Glucagon

This polypeptide hormone (molecular weight 3485 daltons) is produced in the α islet cells of the pancreas and counteracts the actions of insulin. Its principle site of action is believed to be the liver where glycogen is hydrolysed to yield glucose which is released into the bloodstream (Hemms & Whitton, 1980). Glucagon has no effect on glycogen breakdown in muscle whereas adrenaline causes breakdown of glycogen in both muscle and liver. Like adrenaline, glucagon is believed to act through the cyclic AMP protein kinase system. Increased

cyclic AMP levels lead to an increase in the activity of phosphorylase a in the liver through phosphorylation of the enzyme (Exton et al., 1971).

Glucocorticoids

Cortisol is the major glucocorticoid found in man. It is a sterol derivative produced in the adrenal cortex. The principal effect of cortisol is on the process of gluconeogenesis, increasing the rate of synthesis of enzymes responsible for gluconeogenesis. Yorke (1967) reported that the corticosteroid analogue dexamethasone decreased the rate of glucose utilisation in adipose tissue. These results suggested that the maximal rate of uptake was reduced with no change in the concentration of glucose, which gave half maximal uptake of D-glucose. Similar results were observed in rat thymocytes by Junker (1981) who showed that the Vmax for 3.O-methyl-D-glucose transport was reduced in the presence of dexamethasone. The effect of dexamethasone is slow in onset and reaches a maximum only after a two hour preincubation. Makman et al. (1971) reported that the cortisol effect on 2-deoxy-D-glucose uptake in thymocytes was prevented by inhibitors of protein synthesis,and they concluded that protein synthesis was involved in the action of glucocorticoids.

Growth hormone

Growth hormone is a single chain polypeptide hormone of molecular weight 21,500 daltons which is produced by cells in the anterior pituitary. Growth hormone is believed to antagonise the effects of insulin thus decreasing the conversion of glucose to fat in adipose tissue (Bacchus, 1976). Growth hormone has been shown to accelerate the entry of L-arabinose into heart muscle after a 15 minute preincubation (Henderson et al., 1961). This affect was prevented by the

addition of inhibitors of protein synthesis.

In addition to these hormones a number of other compounds have been implicated in the regulation of hexose transport.

Prostaglandins

Fain (1968) demonstrated that prostaglandin E1 increased glucose oxidation and conversion into fatty acids in adipose tissue and isolated adipocytes. The maximal stimulation was about 10% of that produced by insulin. The general finding is that prostaglandins are more potent as anti-lipolytic agents than they are as stimulators of glucose transport.

Prostaglandin E2 has been shown to enhance the effect of insulin on glucose transport in adipocytes but has no effect when added on its own (Olefsky 1977). Olefsky has proposed that prostaglandin E2 is a second messenger for insulin action.

Adenosine

Adenosine has been reported to have insulin-like actions on adipose tissue. Dole (1962) reported that adenosine brought about an increase in the rate of glucose utilisation and oxidation in whole adipose tissue. Adenosine has also been reported to reduce hormone stimulated adenylcyclase activity, intracellular cyclic AMP concentrations and rates of lipolysis (Raben & Matsuzaki, 1966, Fain et al., 1972 and Schwabe et al (1973). Further studies by Taylor & Halperin (1976) suggested that adenosine could also increase the rate of hexose transport. When insulin and adenosine were added together a potentiation of the effect of insulin was observed. Raben & Matsuzaki (1966) suggested

that the effects of adenosine may be due to increased hydrogen peroxide formation due to the oxidation of purine bases by xanthine oxidase. May & De Haen (1979) have also proposed that hydrogen peroxide can stimulate hexose transport in adipocytes, and reported increased catalase activity in insulin treated cells. Therefore direct interpretation of the effects of purine bases upon sugar transport are further confused by possible effects of metabolites.

Malbon et al. (1978) have reported that adenosine and adenosine analogues bind reversibly to two classes of receptors on the adipocyte plasma membrane. These binding sites are not related to adenosine deaminase or the adenosine transport system and may therefore represent specific adenosine receptors analagous to the insulin receptor. Similar purinergic receptors have been reported in smooth muscle by Burnstock (1976) and adenosine and its nucleotides have been proposed to act as neurotransmitters in the sympathetic nervous system.

Adenosine 5' phosphates

Adenosine 5' monophosphate (AMP) and adenosine 5' diphosphate (ADP) were also shown by Dole (1962) to have similar effects to those of adenosine in reducing lipolysis and stimulating glucose oxidation. However higher concentrations of AMP and ADP were required to have a similar effect to adenosine.

Adenosine 5' triphosphate (ATP) has been shown to inhibit insulin stimulated glucose oxidation (Chang et al., 1974). ATP was also effective in reducing the effect of other known activators of hexose transport such as lectins, sulphydryl reagents and spermidine. Exogenous ATP has also been reported to restore insulin responsiveness to

ATP depleted muscles (Yu & Gould, 1978). ATP was found to phosphorylate two low molecular weight plasma membrane polypeptides (Chang et al., 1974), and this phosphorylation was substantially inhibited by the glucose transport inhibitor phloretin. The protein kinase responsible for this phosphorylation was found by Chang et al. to be subject to regulation by cyclic AMP.

Non-physiological modifiers of hexose transport

In addition to these physiological compounds a number of non-physiological compounds and conditions have also been found to cause stimulation of glucose oxidation and transport in isolated adipocytes. Table 6 summarises the major classes of compounds and their effects.

The action of insulin

Possible second messengers in insulin action

The events leading to the activation of hexose transport following the binding of insulin to its cell surface receptor are still unclear. Many intracellular enzymes have been linked to the regulatory influence of cyclic AMP and a number of studies have been directed towards identifying possible second messengers for the action of insulin.

Cyclic AMP

It has been proposed that several of the insulin responsive intracellular enzyme systems such as glycogen synthetase, glycogen phosphorylase (Miller et al., 1975a,b), and the hormone sensitive lipase (Corbin et al., 1970), are regulated by cyclic AMP and the cyclic AMP dependent protein kinase system (Robinson et al., 1972). Insulin has been reported to lower intracellular cyclic AMP levels in adipocytes (Hepp &

Table 6.

Compound	Effect	Reference
Hydrogen peroxide, diamide	Stimulates 3-O-methyl-D-glucose uptake in brown and white fat cells	Czech et al. (1974a) Mukherjee & Lynn 1977 May & de Haen 1979
Sulphydryl reagents	No change in 3-O-methyl-D-glucose transport in unstimulated adipocytes. Sulphydryl blockade prevents the action of insulin	Czech et al.(1974b) Czech (1976b)
Polyamines	Spermine and spermidine stimulate conversion of glucose to CO_2 in isolated adipocytes. The effect is similar to that of insulin.	Lockwood et al. (1971) Lockwood & East (1974)
Lectins	Concanavalin A and wheat germ aglutinin activate glucose transport. These lectins have also been shown to interact with carbohydrate groups on the insulin receptor.	Czech et al., 1974a Czech & Lynn, 1973 Cuatrecasas, 1973 Katzen & Soderman, 1975
Anti membrane antibodies	These antibodies cause insulin-like stimulation of glucose transport rates. Fab fractions are inactive, antibodies must be multivalent. Purified antibody preparations indicate that both anti-insulin receptor antibodies and non-specific anti-membrane antibodies lead to increased hexose transport rates.	Cuatrecasas & Tell (1973) Pillion & Czech (1978) Pillion et al. (1979) Jacobs et al. (1978)
Proteases (trypsin)	Gentle trypsin digestion causes marked stimulation of glucose transport. After 15-20 min. cells return to normal transport rates. All other metabolic parameters are unchanged.	Kono 1969

Table 6 (contd.)

Compound	Effects	Reference
Phospholipase C	Marked increase in glucose metabolism and a loss of insulin sensitivity.	Rodbell 1967 Rosenthal & Fain (1971)
Neuraminidase	Stimulates glucose oxidation and prevents the action of insulin. Neuraminidase has a similar effect to trypsin but is effective at a much lower concentration.	Rosenthal & Fain 1971 Cuatrecasas & Illiano (1971)
Hypertonicity	Stimulates the rate of glucose conversion to CO_2 and the rate of 3-O-methyl D-glucose exit in isolated adipocytes and soleus muscle. Effect very similar to that of insulin.	Clausen et al. (1970)
Mechanical agitation	Stirring or centrifugation cause increases in the rate of transport. Cells recover after stimulation and return to basal rates.	Vega & Kono (1979)

Renner, 1972). This appears to be due to the inhibition of adenyl cyclase (Hepp & Renner, 1972) and the activation of cyclic nucleotide phosphodiesterase activity by insulin (House et al., 1972, and Loten & Sneyd, 1970).

The role of cyclic AMP in the regulation of hexose transport is not clear. There are varying reports on the effects of cyclic AMP when it is added externally to cell suspensions. The general lack of effect of cyclic AMP has been assumed to be due to the inability of such a charged compound to penetrate the plasma membranes of whole cells. Cyclic AMP has however been shown to be present in the bathing medium of whole tissue experiments, and in the perfusates from perfusion experiments (Davoren & Sutherland, 1963; Doore et al., 1975; King & Mayer, 1974 and Nickols & Brooker, 1978). Under some conditions the amount of cyclic AMP released appears to reflect the tissue level (Broadus et al., 1970, Exton et al., 1971).

Studies on the uptake and metabolism of exogenous cyclic AMP in cultured hepatoma cells by Granner et al. (1975) have indicated that the adenosine moiety can enter cells. These experiments do not however rule out extracellular hydrolysis of cyclic AMP to release free adenosine, which is rapidly transported into cells.

Holman (1978; 1979) demonstrated the existence of a facilitated diffusion system for cyclic AMP in the human erythrocyte membrane. Plagemann & Erbe (1977) described the release of a 7-deazapurine analogue of AMP from cultured mouse L cells. This system was apparently an active transport process which was inhibited by ATP depletion of the cells. Rindler et al. (1978) also reported efflux of cyclic AMP from a number of cultured cells. In their experiments Rindler et al. could find no relationship between membrane potential and the rate of cyclic AMP efflux, and they

suggested that cyclic AMP efflux shared many of the characteristics of the organic anion transport system.

In order to overcome the poor penetration of exogenous cyclic AMP the lipophillic derivative N^6-o^2-dibutyryl cyclic AMP was prepared (Henion et al., 1967). This compound was presumed to enter the cell by simple diffusion through the plasma membrane and Henion et al., showed dibutyryl cyclic AMP to be more effective than cyclic AMP in activating lipolysis in fat cells. Corbin & Krebs (1969) showed, however, that dibutyryl cyclic AMP did not stimulate the adipocyte protein kinase. This suggests dibutyryl cyclic AMP must either be metabolised to a compound which can activate protein kinase or it may act via an indirect mechanism such as the inhibition of phosphodiesterase activity. Fain (1973) has reported that dibutyryl cyclic AMP is a poor inbihitor of phosphodiesterase activity in fat cell homogenates. It is difficult to detect the metabolism of dibutyryl cyclic AMP in fat cells since high concentrations are required to elicit effects. A 1% conversion to cyclic AMP would be sufficient to maximally activate lipolysis (Belcher, 1971). The mechanism of dibutyryl cyclic AMP is therefore still unclear. Dibutyryl cyclic AMP has been shown to stimulate glucose oxidation in intact adipose tissue (Bray, 1967 and Schimmel & Goodman, 1971). These results indicated that whilst the effect of dibutyryl cyclic AMP is mainly a stimulation of the enzymes involved in glucose oxidation, Schimmel & Goodman reported that part of the effect was due to a stimulation of hexose transport.

Cyclic GMP

Illiano et al. (1973) reported that insulin elevates the intracellular levels of guanosine 3'5' cyclic monophosphate (cyclic GMP) by three to four fold. Agents such as carbamyl choline which also elevate cyclic GMP levels in adipocytes do not all affect lipolysis (Fain & Butcher, 1976). Furthermore exogenous cyclic GMP or dibutyryl cyclic GMP do not mimic insulin action on glycogen synthesis (Pinkett & Perlman, 1975). 8-bromo-cyclic GMP has however been reported to stimulate hexose uptake in rat diaphragm (Pinkett & Perlman, 1975).

One of the aims of the experiments reported in this thesis was therefore to examine the effects of nucleosides, nucleotides and cyclic nucleotides on the regulation of hexose transport in adipocytes.

Ion fluxes

Insulin has been shown to regulate both anion and cation fluxes in intact cells (Clausen & Hansen, 1977). The effect of insulin on membrane potential and ion fluxes have been shown to be rapid and occur independently of the action of insulin on hexose transport (Czech 1977). Thus, changes in ion transport may lead to activation of the hexose transport system.

Monovalent cations

Since hexose transport in adipocytes is not sodium dependent, direct regulation of hexose transport by sodium ions is unlikely. Letarte & Renold (1967) reported that when sodium ions were not present in the suspending medium the stimulation of glucose oxidation by insulin was reduced. Clausen (1970) suggested that this effect may be due to a sodium requirement for metabolism rather than transport.

In contrast the omission of potassium ions from muscle cell media (Clausen, 1969) or inhibition of the sodium potassium ATPase of isolated fat cells (Bihler, 1968, Bihler & Sawh, 1971a,b) lead to an increase in hexose transport activity. High potassium-content buffers lead to a reduction in the rate of sugar transport (Bihler & Sawh, 1971 b). The intracellular level of potassium ions rather than sodium potassium ATPase activity appeared to modulate these effects. Insulin however leads to opposite effects in the membrane potential with an increased potassium ion concentration inside the cell.

Divalent cations

Several of the effects of insulin on whole cells and the effects of compounds which mimic insulin action have been shown to require calcium ions in the incubation medium (Kissebah et al., 1975). Calcium release from isolated fat cells had been shown to increase with insulin treatment (Martin et al., 1975). Letarte & Renold (1969) observed no effects of high or low calcium concentrations on glucose oxidation by adipocytes. Similarly Rihan et al. (1975) observed no effects on glucose oxidation in the presence of the calcium chelator EDTA. In contrast, Ludvigsen & Jarrett (1980) reported that omission of calcium from the incubation buffer before cell disruption significantly lowered (by 24%) the fossil insulin stimulation of hexose transport observed in plasma membranes. There was no effect on hexose transport in isolated membranes when calcium ions were present or absent.

Vanadium ions have been reported to increase the rate of transport and oxidation of D-glucose in adipocytes (Dubyak & Kleinzeller 1980 and Shechter & Karlish, 1980). Dubyak & Kleinzeller reported that the effect of vanadate was comparable to that of insulin, though vanadate

and insulin together produced no additional effect. The effects of vanadate on hexose transport were observed before significant inhibition of the sodium potassium ATPase occurred. Clausen et al. (1981) also reported effects of vanadate on muscle and adipocytes though they report effects that are somewhat smaller than those exerted by a supramaximal concentration of insulin. Clausen et al. also reported that vanadate stimulated calcium washout from the tissues and suggested that the action of vanadate is related to inhibition of the calcium sensitive ATPase of the endoplasmic reticulum.

Coulston & Dandona (1980) reported that zinc exerts a stimulating action of lipogenesis in adipocytes.

The Jarett peptide

Jarett & Seals (1979) have reported the isolation of a low molecular weight polypeptide which is released from isolated adipocyte plasma membranes on the addition of insulin. This polypeptide is capable of activating the mitochondrial pyruvate dehydrogenase complex. The polypeptide is released by insulinomimetic agents other than insulin (Seals & Jarett, 1980) suggesting that it is not a product of the insulin molecule itself, but rather that it is derived from a plasma membrane component. Reports of the action of this material are so far confined to its action on intracellular enzymes and it has not been studied for effects on the hexose transport system.

Whilst cyclic nucleotides, cellular ion fluxes and other second messengers may be involved in insulin action, these do not give an indication of the mechanism of insulin activation of the hexose transport system.

Models for the regulation of hexose transport

In target tissues insulin and adrenaline both increase the Vmax for hexose transport without affecting the Km. A central problem in the regulation of hexose transport is thus whether or not the actual number of transporters in the plasma membrane is changed by hormonal stimulation. Insulin can either increase the number of functional transporters by conversion from an inactive to an active form or through recruitment of transporters from another cellular compartment to the plasma membrane. Alternatively the Vmax for individual transporters can be changed by insulin. Both these hypotheses are consistent with the observed enhancement of Vmax with no change in the Km. The hypothesis of an increased number of functional transporters suggests that the characteristics of the transporters (e.g. temperature dependence, pH profile and substrate specificity) would be similar in both basal and insulin stimulated cells. The hypothesis of activation of existing transporters by increasing the Vmax suggests that different properties would be exhibited by the transport of basal and insulin stimulated cells.

Studies on the properties of the D-glucose transport system of both basal and insulin stimulated adipocytes suggest that the pH dependence of the transport system is altered by insulin (Whitesell & Gliemann, 1979). In contrast the results of Ludvigsen & Jarett (1980) suggested that the pH profile of D-glucose transport in isolated plasma membranes was similar in plasma membranes from basal and insulin stimulated cells. Whitesell & Gliemann (1979), Amatruda & Finch (1979) and Kono et al. (1977) have all reported differences in the temperature dependence of hexose transport in basal and insulin stimulated adipocytes, although Vinten (1978) and Olefsky (1978) did not report such a difference. Ludvigsen & Jarett (1980) also reported identical temperature dependence for hexose transport in plasma membranes from basal and

insulin stimulated adipocytes.

Differences in the fluidity of the plasma membrane have been proposed as a means of regulating the rate of glucose transport in plasma membranes. Pilch et al. (1980) reported an increase in the rate of glucose transport in plasma membranes from basal adipocytes when cis vaccenic acid or oleic acids were added directly to isolated plasma membranes. Similar results were obtained when these unsaturated fatty acids were added to liposomes containing reconstituted adipocyte hexose transporter proteins. There were no changes observed when these experiments were performed on membranes derived from insulin treated cells. In contrast when stearate was incorporated into membranes from insulin treated cells the transport rate was reduced to basal levels, but there was no effect of stearate in membranes from basal cells.

Insulin has been reported to increase fatty acid desaturase activity (Prasad & Joshi, 1979) and increase phospholipase activity (Bereziat et al., 1978) though no reports of changes in phospholipids following insulin stimulation have been published. Czech (1980) has proposed that insulin either affects bilayer fluidity in localised membrane regions or increases the partition of the hexose transporter in more fluid regions of the plasma membrane.

The observation that multivalent agents such as lectins or anti-membrane antibodies mimic the effects of insulin (Czech et al., 1974a, Cuatrecasas & Tell, 1973) has lead to the suggestion that the initial effect of insulin is to cause aggregation of insulin receptors on the cell surface. Kahn et al. (1978) have reported that the addition of anti-insulin antibodies to adipocytes, submaximally stimulated with insulin, leads to an increased stimulation of transport.

Studies by Jarett & Smith (1977, 1979), using ferritin labelled insulin which can be visualised under the electron microscope, showed that insulin receptors formed clusters which were disaggregated in the presence of cytochalasin B at similar concentrations to that which blocks hexose transport activity. Jarett & Smith (1979) proposed that these clusters formed hexose transport pores through the plasma membrane, cytochalasin B causing inhibition of transport by disaggregating the clusters. Carter-Su et al. (1980) have shown that the insulin receptor and the hexose transporter behave differently as detergent solubilised proteins and that the two activities are distinct polypeptide molecules. This does not rule out the possibility that in the membrane hexose transport protein(s) are associated with insulin receptors by non-covalent means that are disrupted by mild detergents. Thus, aggregation of membrane components appears to be involved in the action of insulin; however the aggregation of insulin receptors is a preliminary step which leads to the activation of the transport system.

The effects of oxidants on whole cells has lead to the proposal by Czech (1977) that membrane redox reactions may be involved in the action of insulin. Czech proposed that the activation of hexose transport involves oxidation of membrane sulphydryl groups to the disulphide form. Studies on isolated plasma membranes by Ludvigsen & Jarett (1980) showed that sulphydryl reagents inhibit both basal and insulin stimulated transport proportionately. Goldstein & Livingston (1978) reported that sulphydryl agents such as diamide affect cell integrity and lead to non-specific uptake. Their results also showed that there was no change in the levels of reduced glutathione in adipocytes when insulin was added. Thus no direct evidence for the thiol redox model has yet been presented.

Wardzala et al. (1978) proposed that the action of insulin is to increase the number of functional transport sites within the plasma membrane. Their studies suggest that insulin treatment increases the number of glucose displaceable cytochalasin B binding sites in the plasma membrane. They have proposed that the glucose displaceable cytochalasin B binding represents binding to the hexose transporter in the adipocyte membrane, and conclude that the action of insulin is to increase the number of functional transport sites in the membrane. In an extension of this study Cushman & Wardzala (1980) showed that glucose displaceable cytochalasin B binding was also present on microsomal membranes of the adipocyte. Their results showed a reciprocal transfer of these cytochalasin B binding sites from the microsomal membranes to the plasma membrane on stimulation with insulin. The total number of sites per cell remained constant on the addition of insulin but the distribution was altered. Karnieli et al. (1981) have further extended this study and have shown the transfer of cytochalasin B binding sites to be reversible and the magnitude of the transfer to be dependent on the insulin concentration. The time course for the transfer of cytochalasin B binding sites is very similar to the time course for insulin activation of hexose transport, showing a similar lag before the initiation of activation. A similar transfer of cytochalasin B binding sites has also been shown by Wardzala & Jeanrenaud (1981) in muscle preparations.

Independently, Suzuki & Kono (1980) proposed a similar mechanism for the action of insulin. They studied glucose transport in liposomes containing extracts of membrane proteins from adipocytes. They were able to extract proteins which catalysed stereospecific D-glucose transport from both plasma membrane fractions and from a low density membrane fraction enriched in UDP-galactose-N-acetyl glucosamine

galactosyl transferase activity, a marker enzyme for the golgi apparatus. When membranes from basal and insulin stimulated cells were compared there was an apparent transfer of the glucose transport activity from the golgi apparatus to the plasma membrane. Kono et al. (1981) have shown that the transfer of transporters was prevented by agents which lowered intracellular ATP. They also showed that protein synthesis inhibitors did not prevent the transfer of transporters.

Thus, two hypotheses are presented for the action of insulin, a direct effect on existing transporters, or the transfer of existing transporters from an intracellular site to the plasma membrane. Since the supporting evidence for both hypotheses is based on indirect evidence, neither hypothesis can at present be regarded as proven.

MATERIALS AND METHODS

The preparation of isolated adipocytes

Rat epididymal adipocytes were prepared by the method of Rodbell (1961) as modified by Gliemann (1967) and Foley et al. (1980a)

Materials

Disposable polystryrene sample containers and polypropylene centrifuge tubes were from Walter Sarstedt Ltd., Leicester. All other vessels were cleaned using Decon-90 and then soaked overnight in 6M hydrochloric acid in order to remove detergent residues. After acid treatment, vessels were thoroughly washed with hot tap water, distilled water and finally, double distilled water. Double distilled water was used to prepare all the buffers used.

Hepes buffer

N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes) was from the Sigma Chemical Co. All other chemicals were BDH Analar grade. The buffer contained:-

Na^+	140mM
K^+	4.7mM
Ca^{2+}	2.5mM
Mg^{2+}	1.25mM
Cl^-	142mM
H_2PO_4/HPO_4	2.5mM
SO_4^{2-}	2.5mM
Hepes	10mM

The buffer was prepared as two 10 x concentrated stock solutions, one containing Na^+, K^+, Ca^{2+}, and Mg^{2+}, and the other contained Hepes and PO_4.

Both these solutions were adjusted to pH 7.6 at 20°C, filtered and stored at 4°C until required. Both solutions were replaced fortnightly.

Preparation of bovine serum albumin

Bovine serum albumin (Fraction V) Lot No. A·4503 from the Sigma Chemical Co. was used. Before use the albumin was extensively dialysed in order to remove any hormones present.

100g of bovine serum albumin was dissolved in 500ml double distilled water and dialysed for 24 hours against 10 volumes of double distilled water at 4°C. The dialysis water was changed at least three times in this period. After dialysis the albumin solution was diluted to give a 10% w/v solution and the pH adjusted to 7.4 by the addition of 1OM sodium hydroxide. The solution was sterilised by filtration, first through a Whatman 41 paper filter, then a Whatman 42 paper filter and, finally, an 0.8μ Millipore filter. This albumin solution was separated into aliquots and then stored at -20°C until required. The albumin stocks were not re-frozen more than once. In order to prepare albumin containing buffers an appropriate quantity of this stock was then added in place of double distilled water during the preparation of the buffers.

Collagenase

Batches of collagenase from Worthington enzymes, the Sigma Chemical Co. and PL Biochemicals were all tested by preparing isolated adipocytes. Criteria for cell viability were the magnitude of the insulin stimulation of lipogenesis from [U-^{14}C]D-glucose, and the release of lactate dehydrogenase activity. Lot No (4197 CLS 40 B) 11P from Worthington enzymes (activity 142 U/mg) gave the largest response to insulin and the lowest rate of lactate dehydrogenase release. This batch

of collagenase was used in all the experiments described.

The preparation of isolated adipocytes

Male Wistar rats weighing between 150-160g, which had been maintained on standard laboratory chow and water ad libit um, were killed by cervical dislocation and the epididymal fat pads were rapidly removed. Typically the pads from two animals were washed in 10mls of 1% albumin Hepes buffer, then minced with scissors (or in some initial experiments minced with a McIlwain tissue chopper into 0.5mM cubes) and transferred to a polystyrene vessel containing 6ml of 3.5% albumin Hepes buffer containing 0.5mg/ml collagenase and 0.5mM D-glucose. The tissue was then allowed to digest, the contents of the vessel being stirred slowly with a teflon coated magnetic stirrer flea. The digestion was terminated when no lumps of undigested material remained; this was normally after approximately 1 hour. The digest was filtered through a nylon mesh (mesh size 250μm) and the adipocytes were allowed to float to the surface over a period of 3-5 minutes. The infranatant buffer was carefully removed with a siliconised pasteur pipette and the cells were gently resuspended in approximately 10ml of Hepes buffer containing 1% albumin. This washing procedure was repeated five times, with one change of the polystyrene vessel holding the cells. The cells were maintained at 37^{o}C throughout the washing procedure. The cell suspension was then adjusted to the required packed cell volume using a micro haematocrit tube. The tube was centrifuged at 1000$_{x}$ g for 15 seconds and the volume of cells and buffer measured.

The preparation of insulin solutions

1mg of recrystallised porcine insulin (Novo) was dissolved in 1ml of 0.03mM HCl and this solution was then diluted with 2ml double distilled water. 1ml of this stock was then diluted to 50ml with 1% bovine serum albumin in Hepes buffer pH 7.4. This stock was stored at -20°C until required. Appropriate dilutions of this 1μM stock solution were then used with cell suspensions.

Submaximal doses of insulin

In order to achieve accurate estimates of the effects of submaximal doses of insulin, cells were suspended in 3.5% w/v albumin Hepes buffer containing 0.05% w/v bacitracin (Sigma Chemical Co.) as described by Whitesell & Gliemann (1979).

The rate of lipogenesis in adipocytes

The rate of conversion of [U-^{14}C]-D-glucose to lipids by isolated adipocytes was estimated in order to assess the hormonal responsiveness of the adipocyte preparation. The method of Dole & Meinertz (1960) was used. [U-^{14}C]-D-glucose was from The Radiochemical Centre, Amersham. All other chemicals were BDH Analar grade.

50μl of an adipocyte suspension at 40% packed cell volume was preincubated for 15 minutes in 500μl of 1% albumin Hepes buffer with or without the test hormone. 10μl of [^{14}C]-D-glucose was then added together with sufficient unlabelled D-glucose to give a final concentration of 1mM. The incubation was continued for 15 to 30 minutes. The preparation was mixed gently every 5 minutes in order to resuspend the cells. The reaction was terminated by the addition of 2.5ml Dole medium (3:2 propan-

1-ol:n'-heptane) and the mixture was shaken vigorously. 1.5ml of n'-heptane was then added and mixed followed by 1ml of distilled water. The phases were allowed to separate and 0.5ml of the n-heptane phase was removed. The radioactivity present was determined by liquid scintillation counting using 5ml of toluene-PPO scintillant (5g PPO l^{-1}).

The estimation of lactate dehydrogenase activity

The release of the cytosolic enzyme lactate dehydrogenase provides a means of measuring the rate of cell lysis. Lactate dehydrogenase activity was estimated by the method described by Bergmeyer & Bernt (1974). The rate of oxidation of NADH by pyruvate was followed by the change in absorbtion at 340nm. NADH (type IV) was from the Sigma Chemical Co. All other chemicals were BDH Analar grade.

An adipocyte suspension was incubated in 1% Albumin Hepes buffer. At different times the cells were allowed to float to the surface and samples of the infranatant were taken. In order to estimate the total lactate dehydrogenase content, whole tissue or isolated cells were lysed in an 0.05% v/v solution of Triton X-100 in distilled water. Samples were stored at $4^{o}C$ before assaying for lactate dehydrogenase.

The assay was carried out at $25^{o}C$ in a 3ml volume containing 48mM sodium phosphate buffer pH 7.5, 0.6mM Na pyruvate, and 0.18mM NADH. in a silica cuvette. The reaction was started by the addition of 50µl of enzyme suspension and the change in absorbance at 340nm was followed by a spectrophotometer.

The preparation of 3'-5'-cyclic AMP binding protein

3'-5' cyclic AMP binding protein was prepared by the method of Tovey et al. (1974) from rabbit skeletal muscle. The entire preparation was carried out at 4°C.

350g of rabbit skeletal muscle was cut into small pieces and homogenised in 500ml of 4mM EDTA (pH 7.0) for 2 min using a Waring Blender. The homogenate was centrifuged at 27,000 x g for 30 minutes. The pellet was discarded and 1.0M acetic acid was slowly added to the supernatant until the pH was 4.8. The mixture was allowed to stir for 10 minutes and then centrifuged at 27,000 x g for 30 minutes. The pellet was discarded and the supernatant brought to pH 6.5 by the addition of 1.0M potassium phosphate buffer (pH 7.0). 32.5g/100ml ammonium sulphate was then added very slowly with continual stirring in order to precipitate the crude binding protein, which was collected by centrifugation at 27,000 x g for 30 minutes. The pellet was dissolved in 6% of the volume before precipitation, of 5mM potassium phosphate buffer (pH 7.0) containing 2mM EDTA and dialysed overnight against three changes of 5mM potassium phosphate buffer containing 2mM EDTA. This yeilded 4.28g of crude binding protein.

The binding protein was further purified by ion exchange chromatography on DEAE cellulose (DE52-Whatman). The DEAE cellulose column (approximately 200ml bed volume) was equilibrated with 5mM phosphate buffer (pH 7.0) containing 2mM EDTA. After the sample was applied in 5mM phosphate buffer + 2mM EDTA the elution was commenced with 0.1M phosphate buffer (pH 7.0) and 10ml fractions of the eluate were collected. After 30 fractions the elution buffer was changed to 0.3M phosphate buffer (pH 7.0) and a further 30 fractions were collected. The entire procedure was carried out at 4°C.

The fractions from the column were assayed for cyclic AMP binding (as described below) and protein was measured by the solution absorbtion at 260 and 280nm. The column elution profile is shown in Fig. 4. Fractions 11-19 gave the optimal cyclic AMP binding characteristics (Tovey et al., 1974) and after being pooled were used for the estimation of cyclic AMP. The prepared binding protein was stored as aliquots at -20°C. The binding properties were found to be stable for at least six months under these conditions.

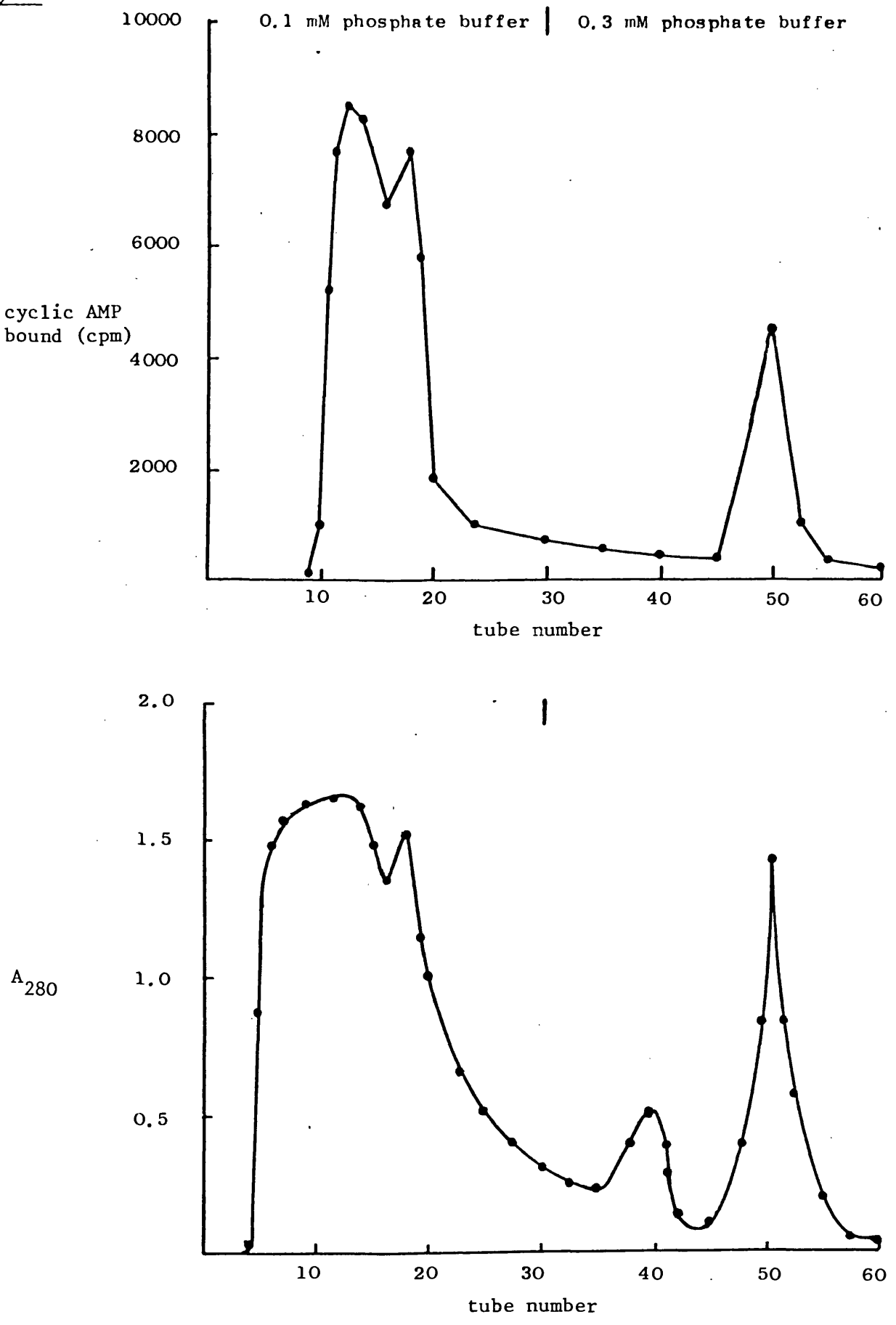
Cyclic AMP binding assay

Cyclic AMP was assayed by the binding assay of Tovey et al. (1974). Norrit GSX charcoal was from BDH, cyclic 3'5' [8-^{3}H] Adenosine monophosphate (30Ci/mMol) was from the Radiochemical Centre Amersham, and Bovine serum albumin and cyclic 3'5' adenosine monophosphate were from the Sigma Chemical Co.

The extraction of cyclic AMP from cells

Cyclic AMP was extracted from whole cells in order for the assay to be performed. 50µl of a 40% PCV cell suspension was incubated in 200µl of 1% albumin-Hepes buffer for the appropriate time, and the reaction was terminated by the addition of 200µl of 5mM tris-HCl buffer + 4mM EDTA (pH 7.4). The samples were immediately placed in a boiling water bath for 5 min. The precipitated protein was removed by centrifugation at 10,000 x g for 4 min and the supernatant was removed. The precipitate was then resuspended in a further 200µl of distilled water, centrifuged, and the supernatants combined. The samples were then lyophylised and dissolved in 100µl of assay buffer. This sample was then assayed for cyclic AMP. Spiked samples gave a 96.3% recovery of added cyclic AMP by this procedure.

Fig 4.



The ion exchange chromatography of crude cyclic AMP binding protein on DEAE cellulose. For details see materials and methods.

The assay of cyclic AMP

The assay was performed in 50mM tris-HCl(pH 7.5) containing 4mM EDTA. The optimal dilution of the stock binding protein (which retained 55% of the added tracer cyclic AMP) was found to be a 1 + 5 dilution of the stock binding protein with assay buffer. The charcoal adsorbent was 520mg of Norit GSX charcoal suspended in 20ml of assay buffer containing 2% w/v bovine serum albumin.

Method

The components of the reaction mixture were added in the following order: 50µl of tritiated cyclic AMP (5µCi of stock isotope in 10ml of assay buffer), 50µl of unlabelled cyclic AMP (standard or unknown) and 100µl of binding protein solution. After mixing the tubes were incubated for 2 hours at $4^{o}C$. 100µl of charcoal suspension was then added to each tube which was then vortex mixed for 10 seconds. The tubes were then centrifuged for 1 minute at 10,000 x g at $4^{o}C$. 200µl of the supernatant (bound fraction) was removed and the radioactivity measured by liquid scintillation counting in 2ml triton:toluene:PPO scintillant ($30:70:5gl^{-1}$). A blank was determined by measuring the cpm in 200µl of supernatant from a tube containing 50µl tritiated cyclic AMP, 150µl assay buffer and 100µl of charcoal suspension. This blank reading was subtracted from all other results.

The cpm bound in the absence of unlabelled cyclic AMP (i.e. the zero dose binding) was designated Co and the cpm bound in the presence of unlabelled cyclic AMP (calibration standard or unknown) was designated Cx. The ratio of Co/Cx was calculated for all the standard cyclic AMP samples and a calibration curve was obtained by plotting Co/Cx against pMoles standard cyclic AMP. The amount of cyclic AMP in the

unknowns was then read off this line using the measured Co/Cx values.

Sources of chemicals for regulation study

Adenosine; adenosine deaminase (from calf intestinal mucosa); 5'adenosine monophosphate; 5'adenosine diphosphate; 3'-5' cyclic adenosine monophosphate; 8-bromoadenosine-3,5,-cyclic-monophosphoric acid; N^6,O^2-dibutyryl adenosine-3'5'-cyclic-monophosphoric acid; 3',5'cyclic guanosine monophosphate; 5' guanosine monophosphate; 8-bromoguanosine-3', 5'-cyclic-monophosphoric acid; 8-bromoinosine-3'5'-cyclic monophosphoric acid; inosine; xanthine; hypoxanthine; guanosine, β nicotinamide adenine dinucleotide; β nicotinamide adenine dinucleotide reduced form (NADH); and chlorpromazine were all from the Sigma Chemical Co.

[8-^{3}H]-adenosine was from the Radiochemical Centre, Amersham. 2'(3') adenosine monophosphate (mixed isomer); uridine; nicotinamide; and 4-acetamido-4'-isothiocyano-stilbene-2-2'-disulphonic acid (SITS) were from BDH Ltd.

5' adenosine triphosphate was from P.L. Biochemicals. 3-aminobenzamide was synthesised by Dr. M.R. Purnell.

Sources of chemicals for the study of the substrate specificity of the adipocyte hexose transporter

1-deoxy-D-glucose; 5-thio-D-glucose; D-galactose; 2-deoxy-D-glucose; D-glucosamine; 2-deoxy-D-galactose; N-acetyl-D-glucosamine; D-talose; L-arabinose; D-xylose; 6-deoxy-D-glucose; D-fucose and phenyl-β-D-glucoside were from the Sigma Chemical Co. 2,3-di-O-methyl-D-glucose; 6-O-methyl-D-galactose; 4,6-O-ethylidene-D-glucose and methyl-β-D-glucose were from Koch Light. D-glucose; methyl-α-D-glucoside, trehalose, and maltose were from BDH Ltd. Cytochalasin B was from Aldrich Chemicals Ltd.

D-[U-^{14}C]-xylose was from the Radiochemical Centre, Amersham. 1-deoxy-D-[6-^{3}H]-glucose; 4,6-O-ethylidene-D-[U-^{14}C]-glucose; n'-propyl-β-D-[6-^{3}H]-glucoside; methyl-β-D-[6-^{3}H]-glucoside, and n'-butyl-β-D-[6-^{3}H]-glucoside were all synthesised by Dr. G.D. Holman.

β-fluoro-D-glucose; 3-deoxy-D-glucose; 3-deoxy-3-fluoro-D-glucose; 3-O-propyl-D-glucose; 6-deoxy-6-fluoro-D-glucose; 6-O-pentyl-D-galactose; 6-O-propyl-D-galactose; n'-propyl-β-D-glucoside and n'-butyl-D-glucoside were synthesised by Dr. G.D. Holman.

Transport assays with whole cells

The rate of hexose transport in whole adipocytes was measured by the method of Whitesell & Gliemann (1979). 3-O-[^{3}H-methyl]-D-glucose and 3-O-[^{14}C-methyl]-D-glucose were synthesised by the method described later, or purchased from the Radiochemical Centre, Amersham or the New England Nuclear Corp. Isotopes from all these sources behaved identically. [3-^{3}H]-D-allose was prepared by Dr. G.D. Holman by the method of Sowa & Thomas (1966) as described by Rees & Holman (1981). Tritiated isotopes were regularly purified by preparative paper chromatography (see p. 100). Phloretin was from K & K Laboratories, unlabelled 3-O-methyl-D-glucose and D-allose were from the Sigma Chemical Co. and the Silicone fluid was Dow Corning 100-200cs supplied by Hopkin & Williams. All experiments were performed at 37°C unless otherwise stated.

Zero trans entry

50µl of an adipocyte suspension, either basal or insulin stimulated at 40% packed cells, was added to 15µl albumin free buffer containing radiolabelled sugar + cold sugar (or inhibitor) in a 3.5ml polypropylene centrifuge tube. Since the bulk of the volume of an adipocyte is a non-aqueous lipid deposit, the cell volume of the adipocytes is not regarded as free solution and the starting concentrations were adjusted to take account of this. D-allose transport was followed over a period of 1, 2 and 3 minutes in insulin stimulated cells, and over a 30 minute period in basal cells. 3-O-methyl-D-glucose transport was followed over 30, 60 and 90 seconds in basal cells and 1, 2 and 3 seconds in insulin stimulated cells. For rapid uptakes, timing was aided by the use of a metronome set to two beats/second. Transport was terminated by the rapid addition of 3ml of 0.33mM phloretin in albumin free Hepes buffer. The phloretin stopping solution was prepared by first dissolving the phloretin

in ethanol (0.2% of final volume), then adding dimethyl sulphoxide (0.08% final volume) and this mixture was rapidly dispersed in albumin free Hepes buffer. A layer of silicone oil was then placed on top of the buffer and the tubes were centrifuged at 2500 x g for 1 minute in a swing-out centrifuge. After centrifugation the cells were removed from the top of the oil layer with a pipe cleaner. The radioactivity associated with the cells was then determined by liquid scintillation counting using 5ml toluene:Triton X-100:ppo scintillant (30:70:5gl^{-1}). Blanks were estimated by adding 1ml of phloretin stopper to the tubes before the addition of the cells. Equilibrium volumes were determined by allowing the cells to equilibrate with tracer for 30-45 min before isolating the cells.

Equilibrium exchange

A similar technique to that described for zero trans entry was used except that the cells were preequilibrated with substrate (and inhibitor) before assaying transport. An adipocyte suspension at 44% packed cells was preincubated for 30 minutes with unlabelled substrate (and inhibitor) so as to give a final cell volume of 40% and the desired substrate (and inhibitor) concentration. These concentrations were adjusted to allow for the non-aqueous volume of the adipocytes.

For both zero trans entry and equilibrium exchange experiments the average rate of uptake was estimated using the integrated rate equation.

$$\frac{V}{S} = \ln \left(\frac{1}{1-f} \right)_t \qquad (8)$$

Where S is the substrate concentration, f is the fractional filling and t is the time of uptake.

Average inhibition constants (Ki's) were derived from the equation

$$\frac{Vo}{V} = 1 + \frac{I}{Ki} \qquad (9)$$

Where Vo is the uninhibited rate, V is the inhibited rate and I is the inhibitor concentration.

For both zero trans entry and equilibrium exchange the non-mediated rates were estimated as that transport which was not inhibited by 50μM cytochalasin B.

Zero trans exit

Cells were prepared to 44% packed cells and radiolabelled substrate (and inhibitor) were added and allowed to equilibrate for 30-45 minutes before the assay. The concentrations were corrected to allow for the non-aqueous volume of the adipocytes and to bring the adipocyte suspension to 40% packed cells.

The exit rate was measured by adding 50μl of the adipocyte suspension to 5ml of albumin free buffer which was magnetically stirred. At the appropriate time transport was terminated by the addition of 5ml of Hepes buffer containing 0.6mM phloretin. 1ml of silicon oil was placed on the surface of the tube, and the tube was centrifuged at 2500 x g for 1 minute. The cells were removed from the surface of the oil using a pipe cleaner and the trapped radioactivity estimated by liquid scintillation counting as described for zero trans entry experiments. In order to determine zero time (blank) values the cells were first treated with 50μM cytochalasin B and then added to 5ml of albumin free Hepes buffer containing 50μM cytochalasin B. 5ml of phloretin stopping solution was then added and the samples treated as described

above. Total exit was determined over a period of 10 minutes. 3-O-methyl-D-glucose exit was measured over 2 and 3 seconds in insulin stimulated cells.

Average exit rates for tracer were calculated using an integrated rate equation similar to equation 8.

$$\frac{V}{S_i} = \frac{\ln\left(\frac{1}{fr}\right)}{t} \qquad (10)$$

where fr is the fraction remaining at time t and Si is the substrate concentration at zero time.

Inhibition constants for the inhibition of tracer exit were calculated using equation 9. The Km for zero trans exit was calculated using equation

$$\frac{Vo}{V} = 1 + \frac{S\ corr}{Km}$$

$$\text{where S corr} = S_i \left(\frac{1 - fr}{\ln\left(\frac{1}{fr}\right)}\right) \qquad (11)$$

Si is the substrate concentration, fr is the fraction remaining, Vo is the rate for tracer (a concentration very much less than the Km) and V is the rate for substrate concentration Si.

The preparation of subcellular fractions of adipocytes

Subcellular fractions of adipocytes were prepared by a modification of the methods of McKeel & Jarett (1970) and Jarett (1974).

Materials

As described for the adipocyte preparation, all vessels used were extensively washed to remove detergent residues. All buffers used were prepared with double distilled water. Sucrose-tris-EDTA buffer contained 0.25M sucrose; 10mM tris-HCl (pH 7.4) and 0.5mM EDTA. After preparation the buffer was filtered and autoclaved then stored at $4^{o}C$ until required. In experiments where Mg^{2+} dependent enzymes were assayed. EDTA was replaced by 0.5mM EGTA. All reagents were BDH Analar grade.

Ficoll-400 was obtained from Pharmacia. Before use a 30% solution of Ficoll in double distilled water was dialysed overnight against 10 volumes of double distilled water, with at least four changes of the dialysis medium. After dialysis the Ficoll was lyophylised and ground to a fine powder.

Method

Homogenisation of the cells

Isolated adipocytes were prepared as described in the previous section. The cells were first preincubated with hormone (if required) for 15 min then washed with 15ml of sucrose-tris-EDTA buffer at $37^{o}C$ (containing hormone if required). The cells were then transferred to a Potter-Elvejhem homogeniser with a clearance of $0.006''-0.010''$. Typically 4mls of cells at 40% packed cells were homogenised in 25ml of sucrose tris EDTA buffer at room temperature by ten strokes of the homogeniser turning at 1,600 rpm. The homogenate was transferred to a

centrifuge tube and the homogeniser washed with a further 25ml of buffer.

The homogenate was then centrifuged at 16,000 x g for 15 min at 4°C. After centrifugation the fat cake was discarded and the supernatant (S1) removed for preparation of the microsomal fraction. The pellet (P1) was resuspended in 50ml sucrose tris EDTA buffer for the preparation of the plasma membrane and mitochondrial fractions.

Preparation of the microsomal fraction

The supernatant (S1), after homogenisation, was centrifuged at 100,000 x g for 70 minutes at 4°C. The supernatant was carefully removed with a Pasteur Pipette (due to the loose pellet formed) and the pellet was resuspended in the required volume to form the microsomal fraction.

Separation of plasma membranes and mitochondria

The resuspended pellet (P1), after homogenisation, was centrifuged at 16,000 x g for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 2ml of sucrose tris EDTA buffer; this material was carefully layered onto 4ml of 6% Ficoll in sucrose tris EDTA buffer in a Beckman SW 50.1 centrifuge tube. This size of gradient could separate the material from 4ml of 40% packed cells without evidence of overloading. The Ficoll gradient was centrifuged at 44,000 x g for 45 min at 4°C. The plasma membrane formed a cloudly layer at the buffer Ficoll interface, which could be removed with a pasteur pipette. The mitochondria and nuclei formed a pellet (P2) at the bottom of the tube. Mitochondria and nuclei were recovered by diluting the pellet with 50ml sucrose tris EDTA buffer in order to wash out the Ficoll and collecting by centrifugation at 16,000 x g for 20 min at 4°C.

The plasma membrane fraction was diluted with 30ml sucrose tris EDTA buffer and centrifuged at 100,000 x g for 70 min. The supernatant was removed carefully without disturbing the loose pellet, and the pellet resuspended in the required volume. This suspension was then sonicated for 30 seconds in a 75W ultrasonic water bath. Once prepared the plasma membrane fraction could be stored overnight in liquid nitrogen, without loss of transport activity.

Marker enzymes for subcellular fractions

Protein was estimated using the method of Lowry et al. (1951) using bovine serum albumin (Fraction V) as standard.

Succinic dehydrogenase (EC.1.3.99.1)

Succinic dehydrogenase activity was assayed by the method of Pennington (1961).

50µl of material was incubated with 0.8ml of: 0.125% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride; 30mM sucrose; 60mM sodium phosphate buffer (pH 7.5) and 50mM sodium succinate at 37°C for 15 min. A blank was performed using similar conditions except that the sodium succinate was omitted. The reaction was terminated by the addition of 1ml of 10% (w/v) trichloroacetic acid in water. 4ml of ethyl acetate was added and after the phases had separated the ethyl acetate layer was removed and 0.3ml of methanol was added. The optical density of the ethyl acetate phase was read at 490nM against an ethyl acetate blank. The molar extinction coefficient of the formazan produced by the reaction is $20.1 \times 10^3 \ M^{-1} \ cm^{-1}$.

NADH oxidase (EC 1.6.99.3)

NADH oxidase activity was measured spectrophotometrically by the method of Avruch & Wallach (1971). NADH and potassium ferricyanide were from the Sigma Chemical Co.

The reaction was carried out in a 1ml silica cuvette containing 0.01mM NADH, 4mM tris-HCl (pH 7.4) and 0.22mM potassium ferricyanide. The reaction was initiated by the addition of 50µl membrane suspension. The reference cuvette contained the same solution but no membrane suspension. The reaction was followed by the change in absorbtion at 340nm by a Pye

Unicam SP-8-100-dual beam recording spectrophotometer.

5' nucleotidase (EC 3.1.5.3)

5' nucleotidase was assayed by the radioenzyme assay of Avruch & Wallach (1971) as modified by Newby et al. (1975).

Purification of [8-^{3}H]-AMP

[8-^{3}H] adenosine 5' monophosphate (The Radiochemical Centre, Amersham) was found to deteriorate on storage. ^{3}H-AMP was purified by ion exchange chromatography on Dowex AG-X100 resin (400-800 mesh, acetate form) in a .5 x 0.5cm column. ^{3}H-AMP was applied as a solution in distilled water and the column washed thoroughly with 20ml distilled water. The ^{3}H AMP was then eluted with 20ml 1M acetic acid, the fractions containing the activity were pooled and lyophylised, then made up to 1mCi/ml with distilled water.

Assay of 5' nucleotidase activity in whole cells

50µl of an adipocyte suspension at 20% packed cells was incubated in a 100µl volume containing: 1mM AMP + [8-^{3}H]AMP; 0.2mg/ml adenosine; 5mM Mg SO_4 and 50mM tris HCl (pH 7.4). The reaction was allowed to proceed for 10 minutes before being stopped by the addition of 200µl of 0.15M zinc sulphate. 200µl of 0.15M barium hydroxide was then added followed by a further 100µl of 0.15M zinc sulphate. The precipitate formed was removed by centrifugation at 10,000 x g for 2 minutes. A 500µl aliquot of the supernatant was removed and the radioactivity was determined using liquid scintillation counting in 5ml of triton toluene ppo scintillant (30:70:5gl^{-1}). Blanks were performed by adding cells to the incubation immediately before the addition of the zinc sulphate. Under these conditions the rate of AMP hydrolysis remained

constant for up to 30 minutes.

Assay of 5' nucleotidase in isolated membrane fractions

50µl of membrane suspension was added to a 200µl volume containing 1mM AMP+ [8-^{3}H]-AMP; 50mM tris-Cl (pH 8.0) and 5mM Mg SO_4. The reaction was allowed to proceed for 30 minutes before being stopped by the addition of 200µl 0.15M zinc sulphate followed by 200µl of Barium hydroxide. The precipitate formed was removed by centrifugation, and 500µl of the supernatant was removed for estimation of the radioactivity as described above. The hydrolysis rate was constant for at least 40 minutes with plasma membrane fractions.

Electron microscopy

Samples of isolated membrane fractions were pelleted by centrifugation then fixed for 1-2 hours in 3% glutaraldehyde in sucrose-tris EDTA buffer (pH 7.4). The pellets were then washed and fixed with 1% Osmium tetroxide for 1 hour. The pellet was washed in sucrose tris EDTA buffer, then dehydrated by passing it through a series of ethyl alcohol/water mixtures until, finally, the sample was washed three times with absolute ethanol.

The samples were embedded in Spurs resin and ultrathin sections cut with a microtome. The sections were stained with lead citrate and uranyl nitrate, before examination under an electron microscope.

Transport assays in isolated membrane fractions

Zero trans entry experiments were performed on isolated membrane fractions by a modification of the methods of Carter et al. (1972) and Ludvigsen & Jarett (1980). All materials were from the sources described previously except for the filters which were from Millipore (UK) Ltd.

10µl of isotopically labelled sugar ([U-^{14}C]-D-glucose or [3-^{3}H]-D-allose) + unlabelled sugar in sucrose EDTA buffer was rapidly mixed with 10µl of membrane suspension. Uptake was terminated after the appropriate time by the addition of 1ml 0.3mM phloretin in sucrose-tris-EDTA buffer. Phloretin was dissolved in sucrose tris EDTA buffers in a similar manner to that described for albumin free Hepes buffer. This mixture was then carefully pipetted into the centre of an 0.22µ pore size Millipore filter under suction on a Millipore filtration apparatus. A further 1ml of stopping solution was then pipetted into the centre of the filter and then the apparatus was washed with a further 10ml of stopping solution. The filter was removed and transferred to a scintillation vial and 1ml of 1% (w/v) trichloroacetic acid in water was added. The trapped radioactivity was estimated by liquid scintillation counting using 10ml of triton:toluene:ppo scintillant (30:70:5gl^{-1}). Blanks were performed by adding the membrane suspension after the addition of the phloretin stopping solution, and the equilibrium volume was determined by leaving samples to equilibrate for 45 min. Non-mediated rates were taken as the transport that occurred in the presence of 50µM cytochalasin B. Average uptake rates were determined by equation 8.

Tests for the purity of sugar analogues

Thin layer chromatography

Two systems were used for the analysis of sugar analogues. Both systems use 0.5mm silica gel plates.

System I Ethyl acetate: Petroleum ether (40-60^{o}bp) 3:2

System II Butanol:ethanol:water 6:3:1

System I was used for the separation of hydrophobic heavily substituted derivatives whilst System II was used for the separation of more hydrophilic derivatives. Material on the plate was visualised by spraying the plate with 5% concentrated sulphuric acid in ethanol and heating to 150^{o}C. This charred organic compounds to produce a darkened area.

Paper chromatography

The sugar analogues were both analysed and prepared by descending paper chromatography on Whatman 3MM paper using butanol: ethanol: water (49:11:19) as the solvent system. Material present on the paper was stained by first soaking in silver nitrate/acetone solution (a few drops of saturated aqueous silver nitrate in $\sim$ 200ml of acetone). The paper was then dried and then soaked in ethanolic sodium hydroxide (a few pellets of sodium hydroxide dissolved in a minimum of water added to $\sim$ 200ml of ethanol).

The synthesis of 3-O-[^{3}H]methyl -D-glucose

3-O-[^{3}H]methyl -D-glucose and 3-O[^{14}C]methyl -D-glucose were synthesised using the same protocol. [^{3}H]-methyl iodide (2.5Ci/mMol) and [^{14}C]-methyl iodide (500mCi/mMol) were from the Radiochemical Centre, Amersham, 1,2-4,6-diisopropylidene-D-glucose was from Koch Light, and all other reagents were BDH Analar grade.

0.25g of 1,2-4,6,-diisopropylidene D-glucose were dissolved in 1.5ml of dried 1,4-dioxan in a round bottom flask. 0.3g of freshly powdered anhydrous sodium hydroxide was added. The breakseal tube containing the radiolabelled methyl iodide was connected and the apparatus evacuated. After breaking the seal the contents were allowed to mix (the methyl iodide being removed from the breakseal by gentle heating) and the flask heated at 60^{o}C overnight with magnetic stirring.

After cooling, the reaction mixture was then added to approximately 100ml of ice water-diethyl ether mixture. The phases were allowed to separate, and the aqueous phase was removed. The aqueous phase was re-extracted with ether and the ether extracts combined. The ether extracts were dried overnight by filtration and the ether and residual dioxan removed by rotary evaporation under reduced pressure. Yield $\sim$ 9mCi 3-O-[^{3}H]methyl -1,2-4,6-diisopropyglidene D-glucose.

The product was dissolved in 1ml ethanol, then stirred for 4 hours at 60-70^{o}C with 0.5g Dowex X8-400 16-40 mesh(H^{+}form). The ion exchange resin was then removed by filtration, and the solvent removed by rotary evaporation. The product was then applied to Whatman 3 M M chromatography paper and the 3-O-[^{3}H]methyl-D-glucose separated by descending paper chromatography using butanol:ethanol:water (49:11:19) as the solvent system. After development of the chromatogram, the radio-active 3-O-methyl glucose was eluted with water and diluted to

0.5mCi/ml with 1% ethanol. The yield was ∿ 8.7mCi.

The synthesis of 6-O-propyl-D-glucose

All reagents were BDH Analar grade except for the 1,2-isopropylidene 3,5-O-benzylidene-glucofuranose which was prepared by Dr. G.D. Holman.

300mg of 1,2-isopropylidene-3,5-O-benzylidene glucofuranose were dissolved in 1.5ml of dried 1,4-dioxan. 0.3g of freshly powdered anhydrous sodium hydroxide and 500µl of 1-iodo-propane were added and the mixture heated at 70°C overnight with stirring.

The reaction mixture was then added to ice water-diethyl ether mixture and the phases were allowed to separate. The aqueous phase was removed, reextracted with diethyl ether and discarded. The combined diethyl ether extracts were then dried with anhydrous sodium sulphate. The sodium sulphate was removed by filtration and the diethyl ether and residual dioxan and 1-iodo-propane removed by rotary evaporation under reduced pressure.

The product was then dissolved in 1ml of ethanol and stirred for 4 hours at 60-70°C with 0.5g Dowex X8-400 16-40 mesh (H^+ form). The ion exchange resin was removed by filtration and the resin washed with distilled water. The water ethanol mixture was extracted with diethyl ether and the ether extract discarded. The aqueous phase was dried by rotary evaporation. The product was applied to Whatman 3.M.M. chromatography paper and developed using butanol:ethanol:water (49:11:19) in a descending chromatography system. After development, the 6-O-propyl-D-glucose was eluted with water, and the water was then removed by

rotary evaporation. The product was then further dried in a desiccator. Yield ∿ 26mg 6-O-propyl-D-glucose.

The synthesis of 6 [(4-nitrobenzyl)-thio]-9-β-D-ribofuranosyl purine (NBMI)

6[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine was synthesised by the method of Brajeswar et al. (1975). Nitrobenzyl bromide and 6-mercaptopurine riboside (6-thio-inosine) were from the Sigma Chemical Co. All other reagents were BDH Analar grade.

100mg of 6 mercaptopurine riboside, 58mg of anhydrous potassium carbonate and 91mg of nitrobenzyl bromide were dissolved in 3ml of dry dimethyl formamide. The reaction mixture was heated at 48-50°C for 2 hours. The mixture was then cooled and poured into 20ml of cold distilled water and the solution adjusted to pH 7 with hydrochloric acid. The gel like precipitate that formed was collected by filtration and washed with cold distilled water. The filtrate was dried by rotary evaporation and extracted three times with 15ml lots of ethyl acetate, and the original precipitate dissolved in the combined extracts. After concentration to 5ml, crystalline material separated out. This product was allowed to crystallise, then washed with cold methanol before being recrystallised from methanol. The product gave a single spot when run on silica gel thin layer chromatograms using butanol:acetic acid:water (6:3:1). The product melted at 196°C. Yield 72mg.

RESULTS

The viability of isolated adipocytes

The isolated adipocyte preparation was first studied using a range of criteria in order to ascertain that the isolated cells showed similar responses to whole tissue. The stability of the cells under the conditions used was also investigated.

Cell stability

Isolated adipocyte suspensions were studied by light microscopy, the cells being stained by the addition of a few drops of 0.05% methylene blue. The cells were of uniform appearance with nuclei visible in some cells. Few free fat globules were visible in fresh preparations. Fat globules can be identified from adipocytes due to the loss of the 'signet ring' appearance of the intact adipocyte and the formation of a uniform droplet. As the preparation aged over a period of hours, more fat droplets became apparent. Samples of adipocyte suspensions at 40% packed cell volume were counted in a haemocytometer, and gave a count of approximately 4×10^6 cells/ml, which is consistent with the figure given by Gliemann (1967) for a similar preparation. The estimation of adipocyte numbers is subject to a large error due to the instability of isolated adipocytes in the glass haemocytometer chamber.

Table 7 shows the results of experiments performed in order to determine the optimal conditions for the collagenase digestion of whole tissue. The release of the cytosolic enzyme lactate dehydrogenase (Ec.1.1.1.27) was used as a measure of the rate of cells lysis. Samples of the suspending medium were taken before and after collagenase digestion, and assayed for lactate dehydrogenase activity. In addition the stimulation of lipogenesis from D-glucose by 10nM insulin was also studied in

Table 7.

Treatment before digestion	None	Tissue minced to 2-4mm cubes (scissors)	1.0mm cubes (McIlwain chopper)	0.5mm cubes (McIlwain chopper)
Lactate dehydrogenase activity in infranatant after treatment (IU)	0.101	0.175	0.106	0.137
Lactate dehydrogenase activity in infranatant after digestion (IU)	0.349	0.729	0.441	0.591
Digestion time (min)	90	60	60	35
Yield of cells (ml at 40% PCV)	0.4	1.3	1.3	1.3
Basal lipogenesis rate (nmol glucose converted/10^5 cells/h)	0.0366	0.029	0.042	0.046
Lipogenesis rate in the presence of 10nM insulin (nmol glucose converted/10^5 cells/h)	1.032	1.088	0.801	1.003
Magnitude of insulin stimulation	28.2	37.5	19.0	21.8

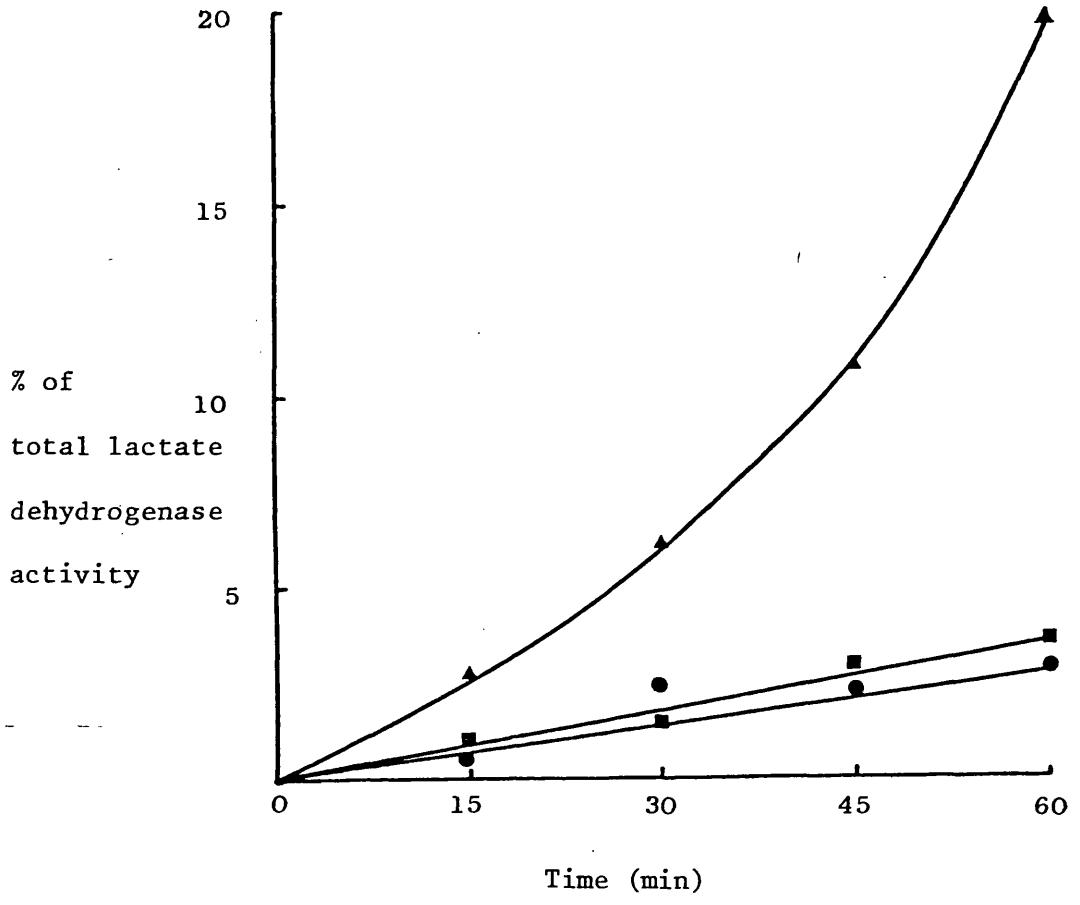
order to measure the sensitivity of the cells to hormones.

Preliminary experiments had shown that 0.5mg/ml collagenase gave optimal digestion conditions. The experiments presented in Table 7 were performed in order to determine the best pretreatment of the tissue. These results indicate that whilst cell damage is caused by mincing the tissue, a longer digestion time is required and a poor yield was obtained by using whole tissue. The optimal procedure which was used for further experiments, was coarse mincing of the tissue with scissors into 2-4mm cubes prior to digestion with collagenase.

The long-term stability of the adipocyte preparation was also studied. Fig. 5 shows the release of lactate dehydrogenase activity into the suspending medium by a 40% packed cell volume suspension of adipocytes. The cells were washed before being resuspended in fresh buffer, and samples of the infranatant were taken after the cells had been allowed to float to the surface. The results show, that for long-term incubations the presence of 1% bovine serum albumin is necessary to prevent cell lysis. Changing the major cation in the buffer (K^+ 140mM, Na 4.7mM, as opposed to the standard buffer which contains Na^+ 140mM, and K^+ 4.7mM,) had little effect on the lysis rate.

The total lactate dehydrogenase content of the isolated cells showed only slight variation between preparations, with a value of 9.08 ± 0.49 IU ml of packed cells (n = 12). A similar variation in activity was seen in whole tissue with an activity of 7.63 ± 0.52 IU/g wet weight (n = 4), though this value may be underestimated due to the difficulty of accurately weighing small amounts of tissue.

Fig 5.

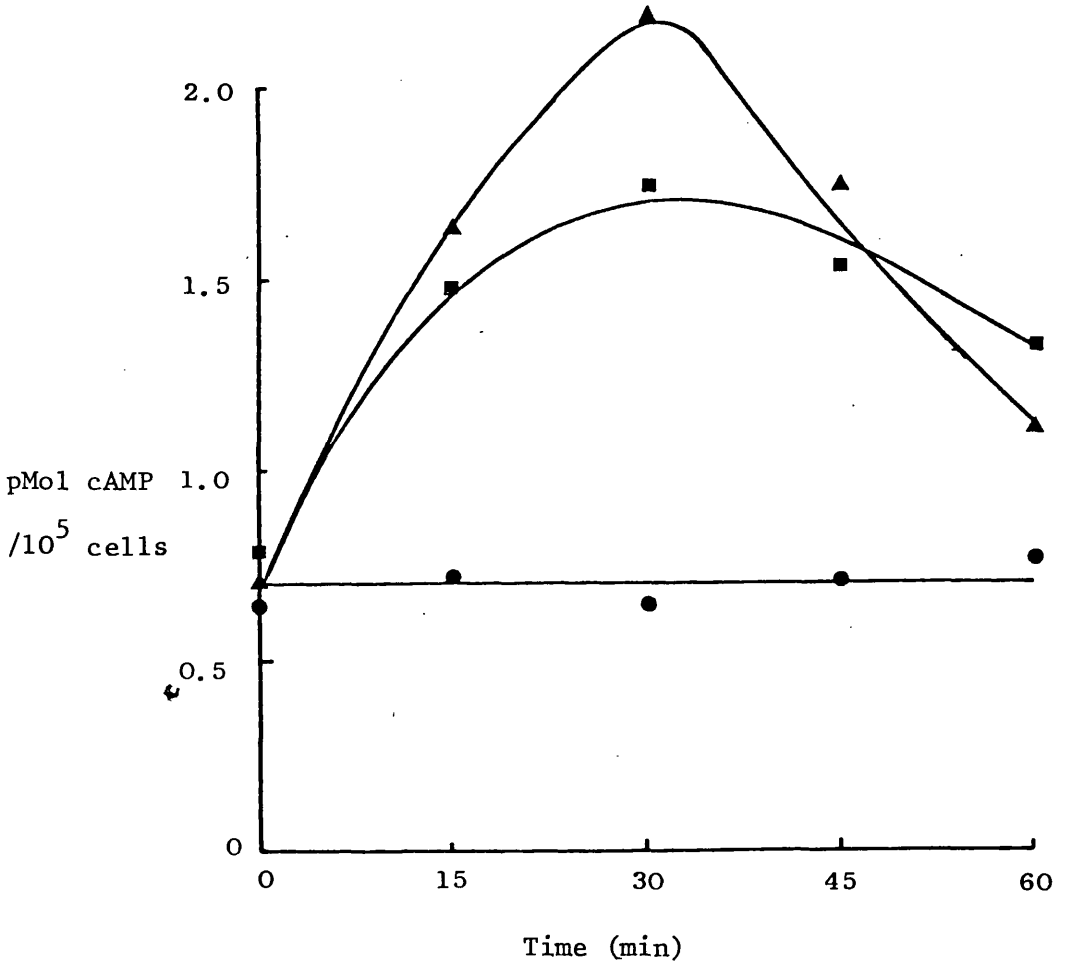


The release of lactate dehydrogenase by isolated adipocytes suspended in (▲) hepes buffer; (●) hepes buffer + 1% bovine serum albumin; (■) high potassium hepes buffer + 1% bovine serum albumin.

Table 7 also shows typical lipogenic responses to 10nM insulin by adipocyte preparations. These values compare well with the 10-20 fold stimulations of lipogenesis shown by whole epididymal fat pads incubated under similar conditions. These results indicate that the response of isolated adipocytes to insulin is similar to that observed before disruption of the tissue. The response of isolated cells to catecholamines was also examined. Fig. 6 shows the changes in cyclic AMP levels in adipocytes on treatment with 5.5μM adrenaline and 10μM isoprenaline. The 1.4 fold increase in cyclic AMP in response to adrenaline is similar to the results of Butcher et al. (1966) who reported a 55% increase in the cyclic AMP content of adipocytes in response to 5μM adrenaline. The rate of accumulation of cyclic AMP is, however, slower than that reported by Birnbaum & Goodman (1977) for whole tissue. The results of Birnbaum & Goodman showed the peak concentration of cyclic AMP to occur within 3 minutes of the addition of 1.4μM adrenaline, with a return to normal levels 20 minutes after stimulation.

Despite the differences observed in the rates of accumulation, these results indicate that the cyclic AMP system of this adipocyte preparation is similar to that reported by other workers.

Fig 6.



The cyclic AMP content of isolated adipocytes following catecholamine stimulation. (●) control; (■) +5.5 μM adrenaline; (▲) + 10 μM isoprenaline.

Characterisation of adipocyte subcellular fractions

Initial attempts to prepare subcellular fractions from rat adipocytes by the hypotonic lysis method of Rodbell (1967) proved unsatisfactory due to the highly conta minated membrane preparation produced. The method of McKeel & Jarett (1970) gave more reproducible results, and is at present used by many other investigators. The homogenisation of the cells is of critical importance to the purity and yield of the final fractions. Vigorous homogenisations resulted in much of the plasma membrane forming very small vesicles. These were similar to the microsomal vesicles and were thus sedimented in the microsomal fractions. More gentle homogenisation leads to the plasma membrane forming larger sacs and sheets of membrane, which can be separated from the microsomal fraction.

The isotonic conditions used in this preparation prevent the rupture of organelles and allow the preparation of intact mitochondria and nuclei. This prevents contamination of the prepared membrane fractions with mitochondrial or nuclear membranes.

The addition of EDTA to the medium was found to be necessary in order to prevent clumping of the membranes and to give reproducible separations. The high concentration used by McKeel & Jarett was however found to be unnecessary, and a lower concentration was found to be effective.

Purity of fractions

The purity of the various fractions was determined by the purity of the characteristic enzyme markers for each fraction (Table 8).

Table 8.

Marker enzyme	Plasma membranes	Mitochondrial fraction	Microsomal fraction
Succinic dehydrogenase µMol/min/mg protein	0.011	0.189	0.001
5'nucleotidase µMol/h/mg protein	2.479	0.170	0.398
NADH oxidase µMol/min/mg protein	No activity	No activity	9.65

Succinic Dehydrogenase

Succinic dehydrogenase activity was chosen as a marker for the mitochondrion, since it is found exclusively in this organelle, tightly bound to the inner membrane (Bachmann et al. 1966). As Table 8 shows this activity is mainly found in the mitochondrial fraction, with only slight contamination of the plasma membrane fraction.

NADH oxidase

This enzyme was chosen as a marker for the microsomal fraction. Avruch & Wallach (1971) reported that NADH oxidase activity is found in both the microsomal and the mitochondrial fractions. A rapid potassium ferricyanide independent reaction was observed in the mitochondrial fraction, with no increase in the rate of NADH oxidation being observed on the addition of potassium ferricyanide. Table 8 shows the activity of this enzyme in the fractions produced, with no activity being detected in the plasma membrane fraction.

5' nucleotidase

5' nucleotidase has been used as a marker enzyme for the plasma membrane fraction by many other workers (Avruch & Wallach (1971); Kono et al. (1977) and Cushman & Wardzala (1980)). Table 8 shows that this activity is mainly recovered in the plasma membrane fraction, with small amounts in the mitochondrial and microsomal fractions. This activity may represent plasma membrane contamination of these fractions.

In order to determine the orientation of the prepared plasma membranes, the presence of latent 5' nucleotidase activity was investigated. De Pierre & Karnovsky (1973) showed histochemically that 5' nucleotidase is an exoenzyme located at the plasma membrane with the

active site facing the external solution. Since AMP is a charged molecule it cannot penetrate the cell membrane, and is therefore only available to 5' nucleotidase activity which is on the outside face of membrane vesicles, i.e. those plasma membrane vesicles which are 'right side out'. The addition of a low concentration (0.05% v/v) of the non-ionic detergent Triton X-100 leads to disruption of vesicles, allowing enzyme (which is isolated from its substrate in 'inside out' vesicles), access to the substrate. The plasma membrane 5' nucleotidase activity increase from 2.48 μMol/h/mg protein to 4.57 μMol/h/mg protein in the presence of 0.05% v/v Triton X-100, whereas the activity in the microsomal fraction increased from 0.40μMol/h/mg protein to 0.51μMol/h/mg protein in the presence of detergent. These results suggest that the plasma membrane preparation is a mixture of approximately 1:1 'right side out' and 'inside out' vesicles.

A similar approach was used on isolated adipocytes in order to investigate whether all the 5' nucleotidase activity is accessible at the cell surface. Homogenisation of isolated adipocytes in 0.05% Triton X-100 leads to a doubling of the 5' nucleotidase activity. The activity expressed by whole cells was 25.72 ± 0.17nMol/h/20μl packed cells, (n = 11) whilst after homogenisation the activity was 53.67 ± 5.13nMol/h/20μl packed cells (n = 3). This result is similar to that reported by Stanley et al. (1980) who reported a 20-50% increase in activity in a similar experiment. Thus 5' nucleotidase may not be a satisfactory marker enzyme for the plasma membrane because of the possibility that activity may also be present in intracellular membranes.

Electron microscopy

In order to investigate the morphological characteristics of the membrane fractions prepared from adipocytes, samples of the membrane fractions were examined by electron microscopy.

Plasma membrane

The plasma membrane fraction consisted of large membranous sacs with numerous invaginations and microvesicles (Fig. 7). There was little evidence of mitochondrial contamination, the major contaminant being microsomal membranes similar to those shown by McKeel & Jarett (1970). As can be seen from Fig. 7 microsomal membranes were often trapped within large vesicles.

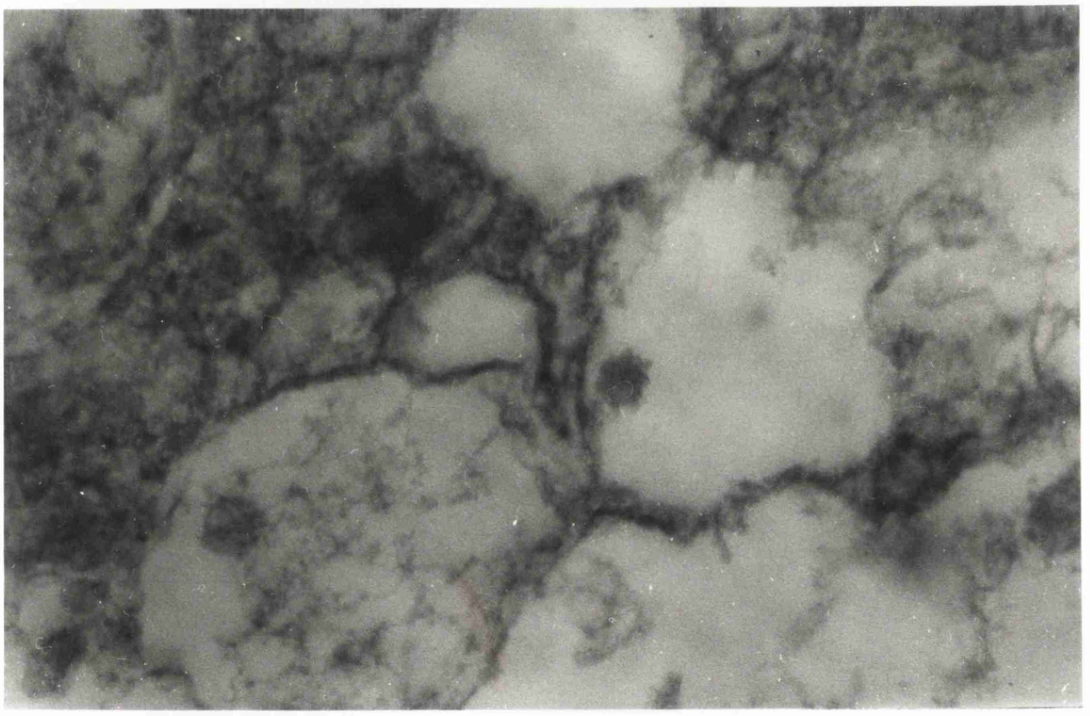
Mitochondria

The mitochondrial fraction appeared to contain mostly swollen mitochondria with clearly descernible cisternae. The outer membrane was still present in most cases (Fig. 8). The mitochondrial fraction was contaminated with the occasional intact nucleus and some small membrane vesicles. Glycogen aggregates were also occasionally observed in this fraction.

Microsomal fraction

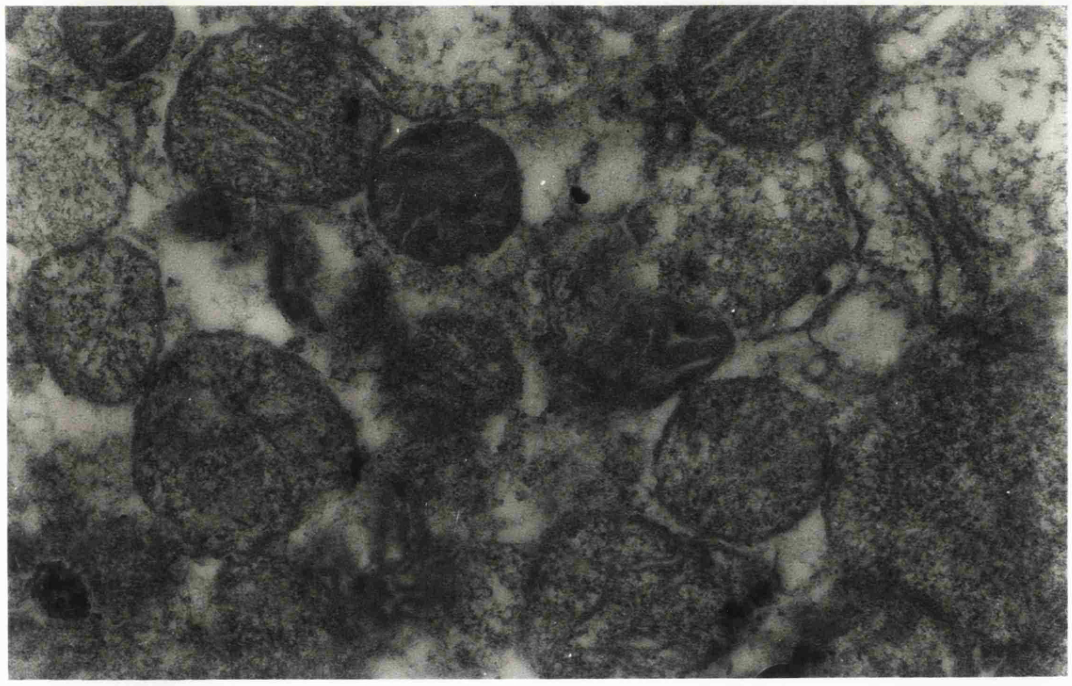
The microsomal fraction consisted mainly of closed vesicles considerably smaller than those observed in the plasma membrane fraction. There was also a considerable amount of unidentified densely stained material present (Fig. 9).

Fig. 7.



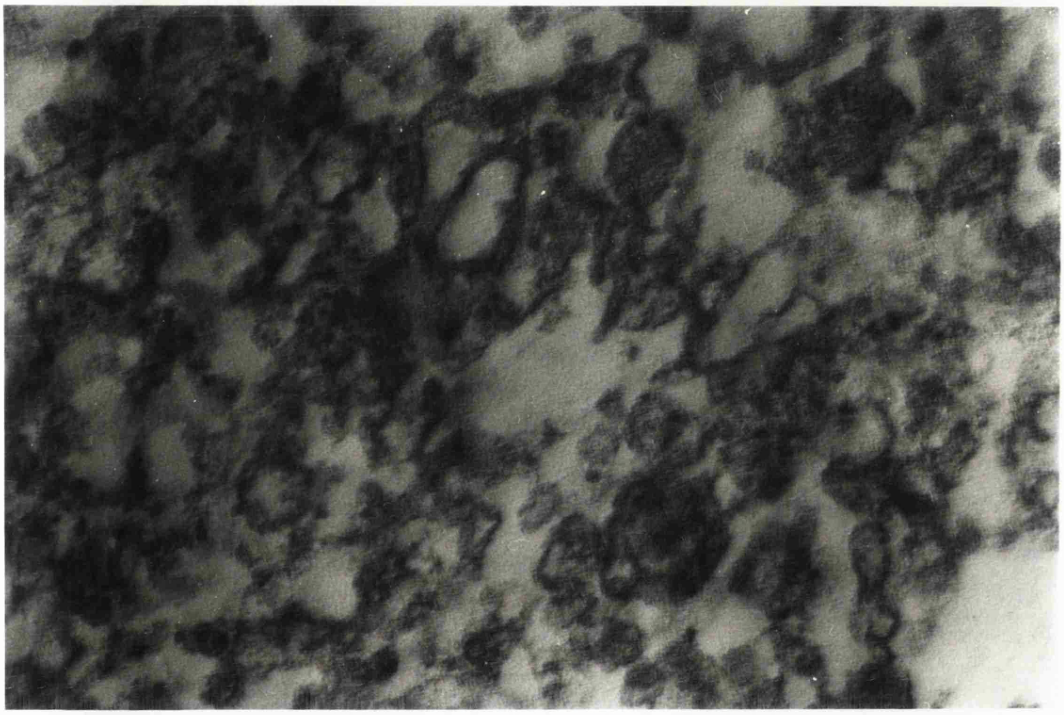
Plasma membrane vesicles from a Ficoll gradient showing sacs of plasma membrane. One sac contains material resembling endoplasmic reticulum. x 33,000.

Fig. 8.



Mitochondrial fraction from bottom of ficoll gradient. The mitochondria are swollen but most appear to retain the outer membrane. Small vesicles are also present. x 25,000.

Fig. 9.



Microsomal fraction showing small closed vesicles and some membrane fragments. x 63,000.

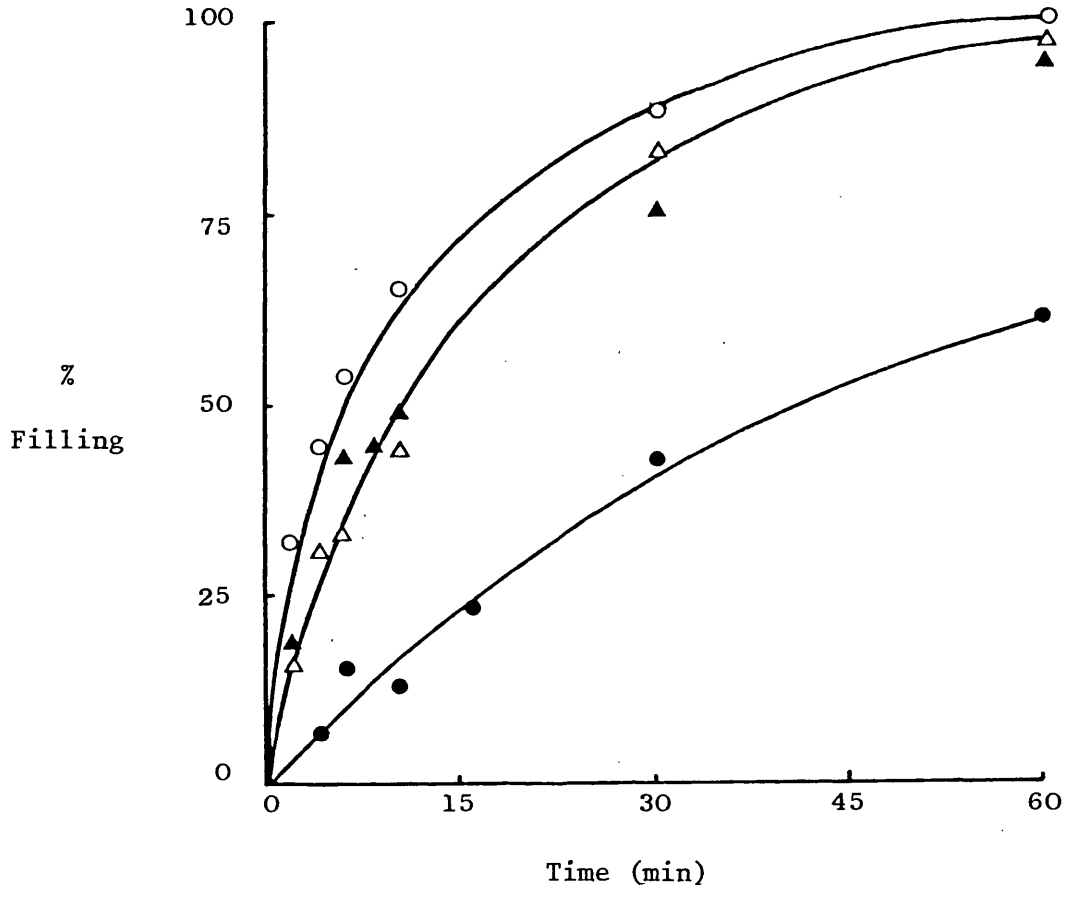
Hexose transport in subcellular fractions

D-glucose displaceable cytochalasin B binding to the microsomal fraction of adipocytes has been shown by Cushman & Wardzala (1980), and stereospecific D-glucose uptake has been demonstrated in liposomes containing protein extracts of microsomal membranes by Suzuki & Kono (1980). Martin & Carter (1970) demonstrated D-glucose transport in isolated plasma membranes and showed that insulin pre-stimulation of D-glucose transport persists in the isolated membranes.

Fig. 10 shows the uptake of 5mM D-glucose in subcellular fractions of both basal and insulin stimulated cells. In plasma membranes the trapped volume is equal to 0.82µl/mg protein in membranes prepared from both insulin stimulated and basal cells and remains constant for at least 30 minutes, indicating that no incorporation of the label is occurring. The rate constant for 5mM D-glucose uptake in plasma membranes prepared from basal cells is 0.024 ± 0.005 sec^{-1} ($n = 8$) whereas in plasma membranes prepared from insulin stimulated cells the rate constant is 0.117 ± 0.022 sec^{-1} ($n = 8$), showing that the rate is increased by insulin. The rate constant for 5mM D-glucose uptake into microsomal membranes is 0.069 ± 0.032 sec^{-1} ($n = 6$) for membranes from basal cells, and for membranes from insulin stimulated cells it is 0.068 ± 0.008 sec^{-1} ($n = 6$) indicating no change in the rate of uptake when the cells were pretreated with insulin. The trapped volume of the microsomal fraction is 0.61µl/mg protein in microsomes from both basal and insulin stimulated cells.

The rate constant for the uptake of 5mM D-allose in adipocyte plasma membranes is $0.38 \pm 0.15 min^{-1}$ ($n = 4$) for membranes prepared from insulin stimulated cells. The trapped volume of D-allose is 0.84 µl/mg membrane protein which is similar to that for D-glucose.

Fig 10.



A time course for the uptake of 5mM D-glucose in (●) plasma membranes isolated from basal cells; (○) plasma membranes isolated from insulin stimulated cells; (▲) microsomal membranes isolated from basal cells; (△) microsomal membranes isolated from insulin stimulated cells.

The rate constants for 5mM D-allose uptake in the microsomal fraction of both basal and insulin stimulated cells are similar at 0.55 ± 0.38 min^{-1} (n = 4) for basal cells and 0.54 ± 0.20 min^{-1} (n = 4) for insulin stimulated cells. This rate is similar to the rate for D-glucose uptake in the microsomal fraction.

The magnitude of the stimulation of hexose transport in plasma membranes by 10nM insulin is less than that seen in whole cells (see p. 121). However, this is consistent with the observations of other workers using a similar preparation. The membrane system also shows differences in the relative stimulation by insulin of D-glucose and D-allose transport. A similar difference in the relative stimulation of 3-O-methyl-D-glucose and D-allose transport is observed in whole cells when the rate due to non-mediated uptake is not subtracted. The non-mediated uptake can be measured by blocking mediated uptake with 50μM cytochalasin B (see p. 124). The difference in the relative insulin stimulation is thus presumed to be due to a non-mediated component in the transport of hexoses into membrane vesicles.

The facilitated diffusion of hexoses in plasma membranes from adipocytes can be completely blocked by 50μM cytochalasin B (Ludvigsen & Jarett, 1980). The rate of D-glucose transport in microsomal membranes is however unaffected by 50μM cytochalasin B. There is also no change in the rate of D-glucose transport in microsomal membranes over a range of 1 to 20mM D-glucose.

The uptake of D-glucose by microsomal membranes does not show saturation kinetics, stereospecificity or inhibition, indicating that the uptake of hexoses into these fractions occurs by a non-mediated route.

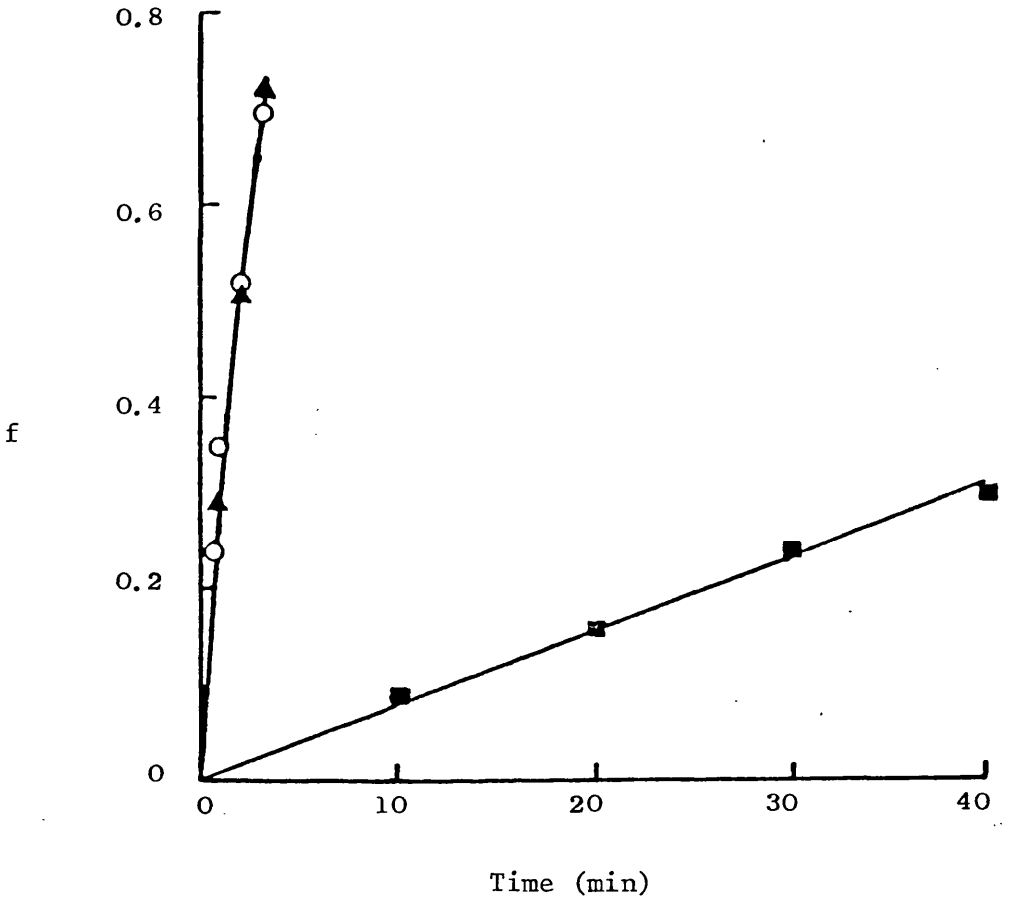
The transport of D-allose in adipocytes

Fig. 11 shows the uptake of 1.3mM D-allose in adipocytes treated with 10nM insulin. The uptake follows a simple exponential function of time, and fractional fillings of up to 75% gave linear plots vs time using the integrated rate equation (see p. 90). Fig. 11 also shows the efflux of D-allose from adipocytes which had been preincubated for 45 min with tracer D-allose. The rates of influx and efflux are equal, indicating that at equilibrium D-allose is in free solution within the cell and non-metabolised. The equilibrium volume of D-allose is similar to the 3-O-methyl-D-glucose equilibrium volume and equal to 1.8µl/100µl packed cells. These results are consistent with D-allose being transported but not metabolised.

In the presence of insulin the transport of 1.3mM D-allose gives a $t_{\frac{1}{2}}$ of 1.5 minutes, whereas in the absence of insulin the rate is approximately 50 times slower with a $t_{\frac{1}{2}} \simeq$ 75 minutes. In basal cells, D-allose transport cannot be completely inhibited by 50µM cytochalasin B, or D-glucose. If this non-mediated rate is subtracted from the total uptake rate the increase in mediated transport on insulin stimulation is identical to that observed when 3-O-methyl D-glucose is used as the substrate. The ratio of permeabilities for 3-O-methyl-D-glucose in basal and insulin stimulated cells is similar to that observed for D-allose and equal to 44.

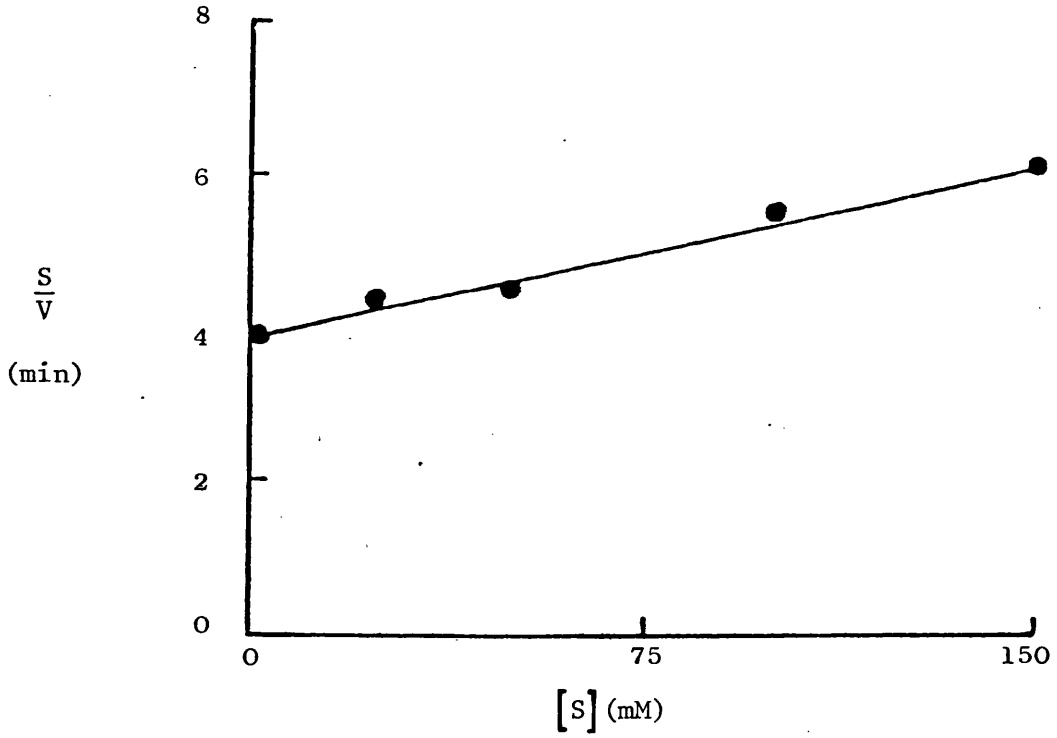
The kinetic parameters for zero trans entry of D-allose in insulin treated adipocytes (Fig. 12) show K_{zt}^{oi} = 271.3 ± 34.2mM and V_{zt}^{oi} = 68.7 ± 7.3mMmin^{-1}. The Vmax for D-allose is similar to that for the zero trans entry of 3-O-methyl-D-glucose in insulin stimulated

Fig 11.



Time courses for the fractional efflux (O) and fractional influx (▲) of 1.3 mM D-allose in adipocytes treated with 10 nM insulin. In insulin treated cells the influx $t_{\frac{1}{2}}$ = 1.77 ± 0.13 min (n = 12). In basal cells (■) $t_{\frac{1}{2}}$ = 75.88 ± 4.99 min (S.E., n = 7).

Fig 12.



Zero trans influx of D-allose in adipocytes treated with 10 nM insulin. K_{zt}^{oi} = 271.3 ± 34.2 mM, V_{zt}^{oi} = 1.15 ± 0.12 mM sec^{-1}. (Best fit estimates ± S.E. from weighted regression, n = 5)

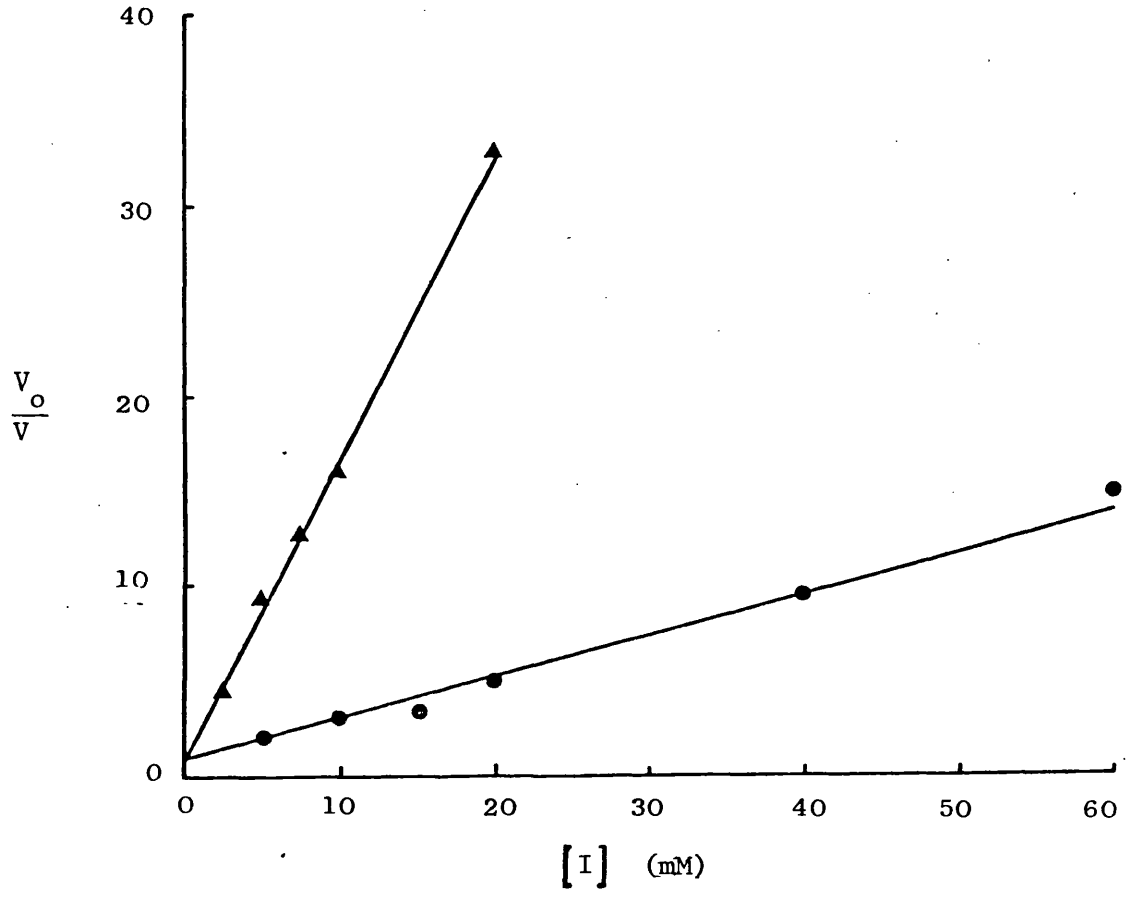
adipocytes reported by Taylor & Holman (1981). The Km for D-allose is much higher and therefore, at the concentration of D-allose used in the inhibition studies, the D-allose permeability is very low since the concentration of substrate (1.3mM) is much lower than its Km. Under these circumstances the permeability is given by the V/K ratio.

In insulin-stimulated cells the non-mediated uptake of D-allose is a small component of the total uptake. This did not significantly influence the inhibition plots until the mediated uptake of D-allose was significantly inhibited (at $\frac{Vo}{V}$ values greater than 6) when the inhibition plots tended to form a curve. By subtracting the non-mediated D-allose uptake (the rate in the presence of 50μM cytochalasin B) $\frac{Vo}{V}$ vs I plots remained linear. Fig. 13 shows the inhibition of D-allose uptake by 3-O-methyl-D-glucose and 2-deoxy-D-glucose corrected for non-specific uptake. In both cases the inhibition plots remain linear up to the practical limits of the experiment.

Fig. 14 shows the equilibrium exchange of 3-O-methyl-D-glucose in insulin-stimulated adipocytes. The experiment shows a $K_{ee}^{oi} = 4.54 \pm 0.59$mM and a $V_{ee}^{oi} = 0.82 \pm 0.04$ mM sec^{-1}, which is similar to the value given by Taylor & Holman (1981) of $K_{ee}^{oi} = 4.45$mM and $V_{ee}^{oi} = 0.84$mM sec^{-1} measured over a lower concentration range. There is no evidence in the adipocyte for the non-linearity in reciprocal plots, observed by Holman et al. (1981) in the human erythrocyte.

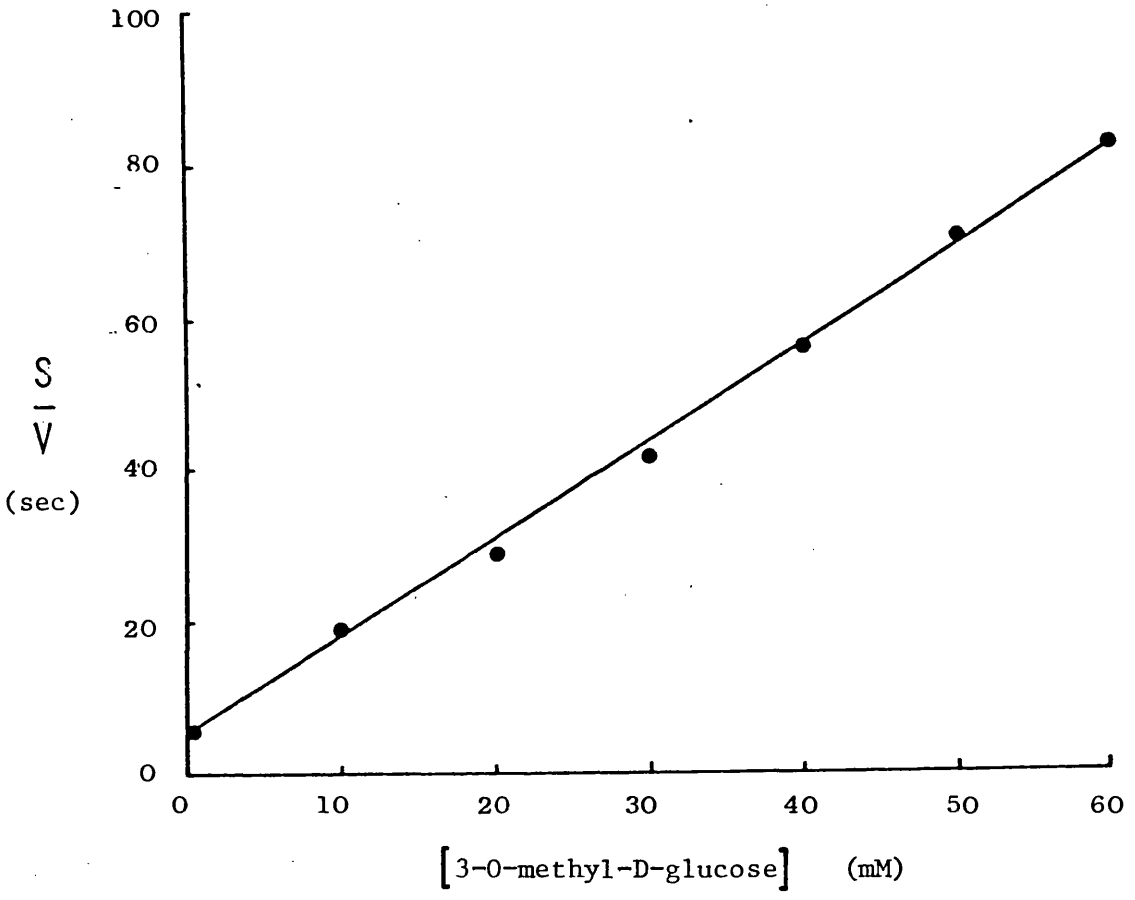
The Km for 3-O-methyl-D-glucose exchange is very similar to the Ki for 3-O-methyl-D-glucose inhibition of D-allose exchange suggesting that the Ki gives a good measure of the Km.

Fig 13.



The inhibition of D-allose exchange (in adipocytes stimulated with 10 nM insulin) by 2-deoxy-D-glucose (▲), K_i=0.74± 0.03 mM (S.E., n=10); and 3-O-methyl-D-glucose (●), K_i = 4.75 ± 0.36 mM (S.E., n=18).

Fig 14.



The equilibrium exchange of 3-O-methyl-D-glucose in adipocytes treated with 10 nM insulin. K_{ee}^{oi} = 4.54 ± 0.59 mM, V_{ee}^{oi} = 0.82 ± 0.04 mM sec^{-1}. (Best fit estimates ± S.E. from weighted regression, n= 7).

Hydrogen bonding requirements for the insulin sensitive hexose transporter of the rat adipocyte

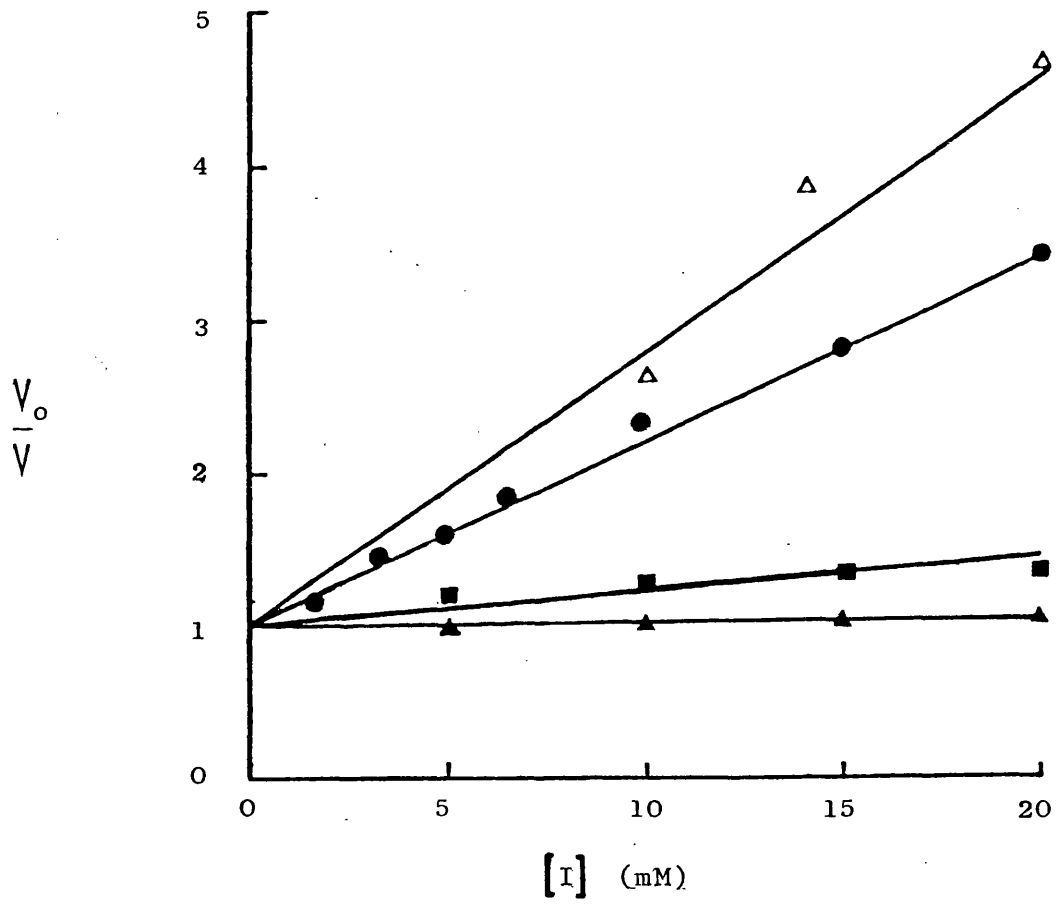
The hydrogen bonding requirements of the adipocyte hexose transporter were examined by studying the inhibition of D-allose exchange by a range of D-glucose analogues. The relative Ki's of the analogues give a measure of the affinity of the analogue for the hexose transporter. Due to the effects of metabolism (see p.161) the comparisons were confined, as far as possible, to studies involving poorly metabolised D-glucose analogues. All analogues were equilibrated with the cells for 30 min before the transport assay was performed, so that the analogues were on both sides of the membrane.

C-1 analogues

D-glucose had a Ki of 8.62 ± 0.71mM for the inhibition of D-allose exchange in adipocytes. Removal of the C-1 hydroxyl to produce 1-deoxy-D-glucose gave a large reduction in affinity with no detectable inhibition over the concentration range studied (Fig. 15). A C-1 hydroxyl is not essential for transport however. Fig. 16 shows the uptake of 1-deoxy-D-glucose by insulin treated adipocytes in the presence and absence of D-glucose. At the concentration used uptake is slow, but D-glucose inhibits the uptake showing that 1-deoxy-D-glucose is transported by the D-glucose transport system. The initial rate of uptake for 1-deoxy-D-glucose in the absence of D-glucose is 20mM min^{-1}. Assuming a Vmax for 1-deoxy-D-glucose of 60-80mM min^{-1} (the Vmax for both D-allose and 3-O-methyl-D-glucose) then the approximate Km for 1-deoxy-D-glucose is > 150mM.

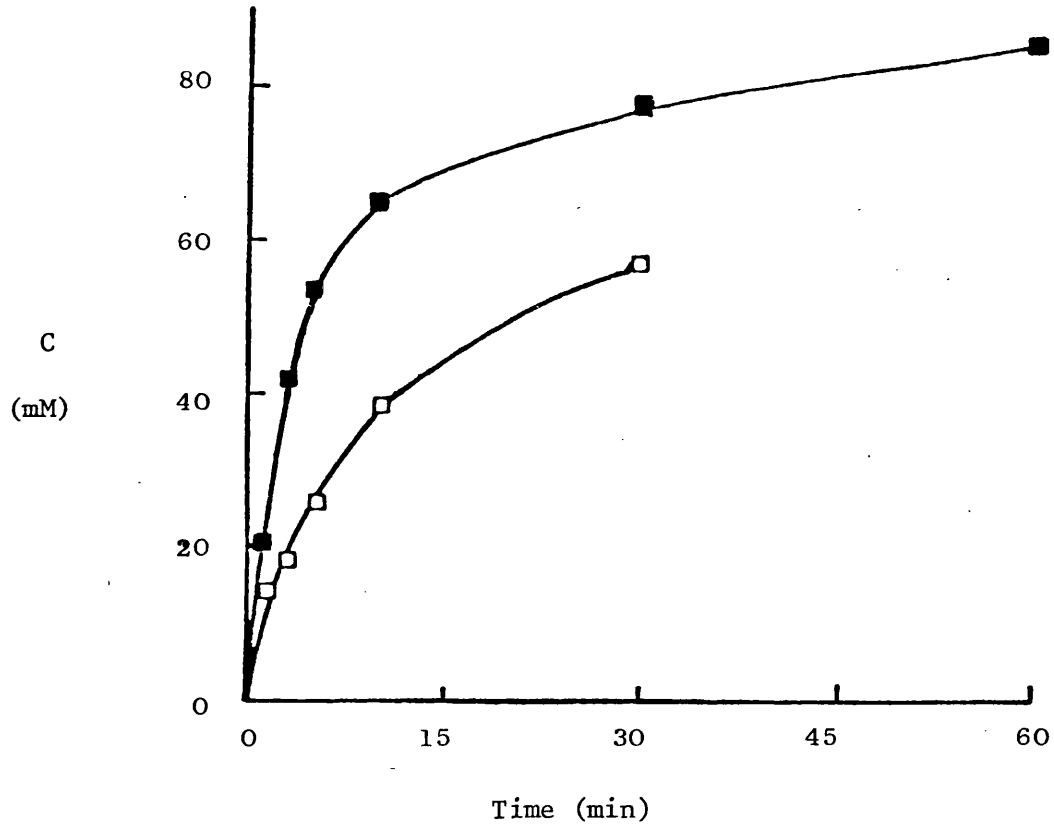
Fluoro substitution of hydroxyls has previously been used by Barnett et al. (1973a) to indicate hydrogen bonding. β-glucopyranosyl fluoride is a good inhibitor with a Ki = 6.87 ± 0.59mM (Fig. 15). The

Fig 15.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-glucose analogues modified at C-1. (▲) 1-deoxy-D-glucose, K_i > 150 mM; (■) 5-thio-D-glucose, K_i = 42.1 ± 6.0 mM (S.E., n=12); (●) D-glucose, K_i = 8.62 ± 0.71 mM (S.E., n=34); (△) β-D-glucopyranosyl fluoride, K_i = 6.87 ± 0.59 mM (S.E., n=18).

Fig 16.



A time course for the uptake of 85 mM 1-deoxy-D-[6-^{3}H]-glucose in cells treated with 10 nM insulin in the presence (□) and in the absence of (■) 50 mM D-glucose. Results are the means of duplicate observations.

electronegative fluorine atom appears to accept a H group from the transporter with no loss of affinity when compared to the hydroxyl of D-glucose. This indicates that the electronegative oxygen group of the hydroxyl probably forms a hydrogen bond with an electropositive group from the transporter. The hydrogen atom of the hydroxyl group does not appear to form a significant hydrogen bond with an electronegative group on the transporter.

Substitution at C-1 of D-glucose results in a fused pyranose ring form. The results of C-1 substitution indicate that the pyranose ring is accepted with high affinity by the site (β-glucopyranosyl fluoride) and transported (1-deoxy-D-glucose), therefore the open chain form of the sugar molecule is not required.

The ring oxygen

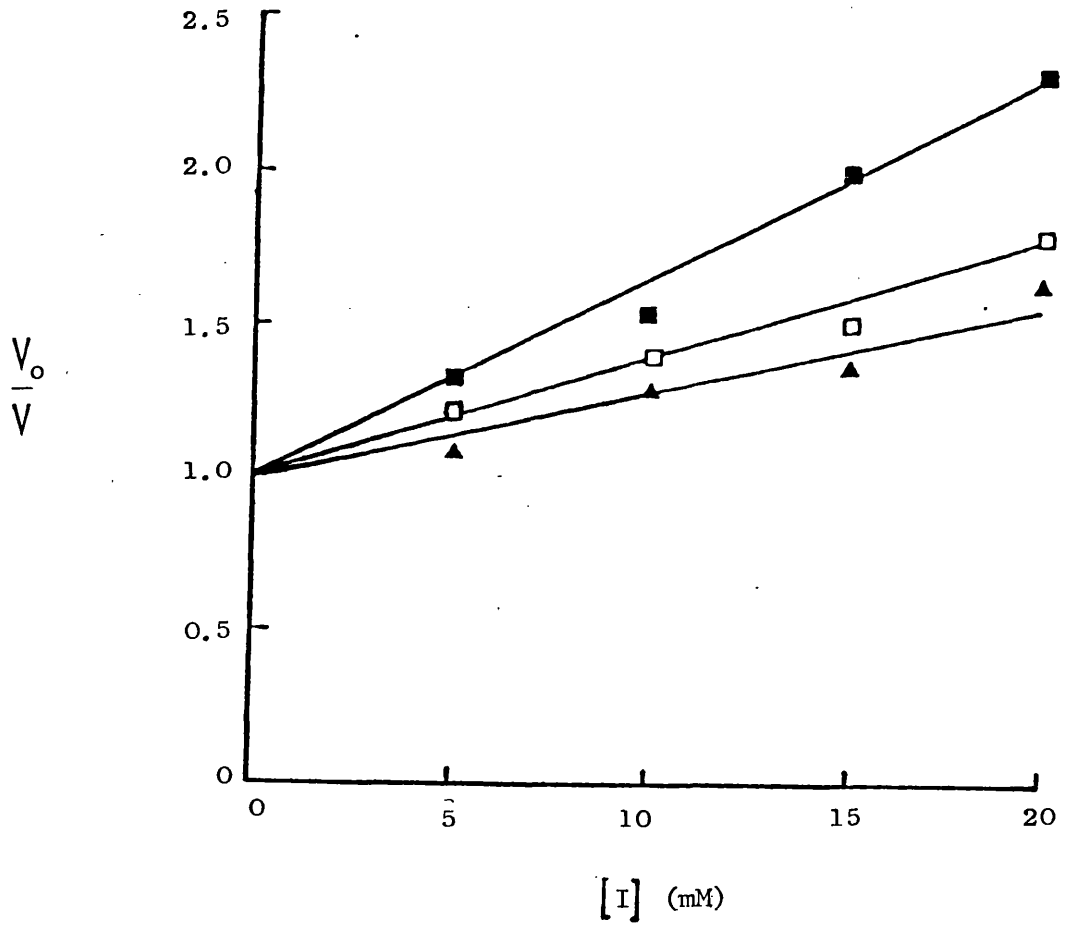
Replacing the ring oxygen with a ring sulphur results in a large loss of affinity. The Ki for 5-thio-D-glucose is 42.1 ± 6.0mM (Fig. 15). This indicates that a hydrogen bond is formed from the transporter to the electronegative ring oxygen.

C-2 analogues

The effect of modifications at the C-2 position of the D-glucose molecule is shown in Fig. 17. Because of the high affinity of 2-deoxy-D-glucose and D-mannose for hexokinase and the effects of metabolism on sugar transport (see p.161) the study was confined to D-galactose analogues modified at C-2.

The Ki of 2-deoxy-D-galactose (20.75 ± 3.04mM) is slightly less than that of D-galactose (Ki = 24.29 ± 3.05mM). This indicates that there is no hydrogen bond directed towards the C-2 hydroxyl and

Fig 17.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-galactose analogues modified at C-2. (▲) D-talose, K_i = 35.4 ± 5.3 mM (S.E., n=10); (□) D-galactose, K_i = 24.49 ± 3.05 mM (S.E., n=12); (■) 2-deoxy-D-galactose, K_i = 20.75 ± 3.04 mM (S.E., n=9).

that a gluco-configuration hydroxyl at C-2 interferes with binding. D-talose, the D-galactose analogue with a manno configuration hydroxyl at C-2, has a Ki = 35.4 ± 5.3mM indicating that a manno configuration hydroxyl at C-2 interferes with binding to a greater extent than a gluco-configuration hydroxyl.

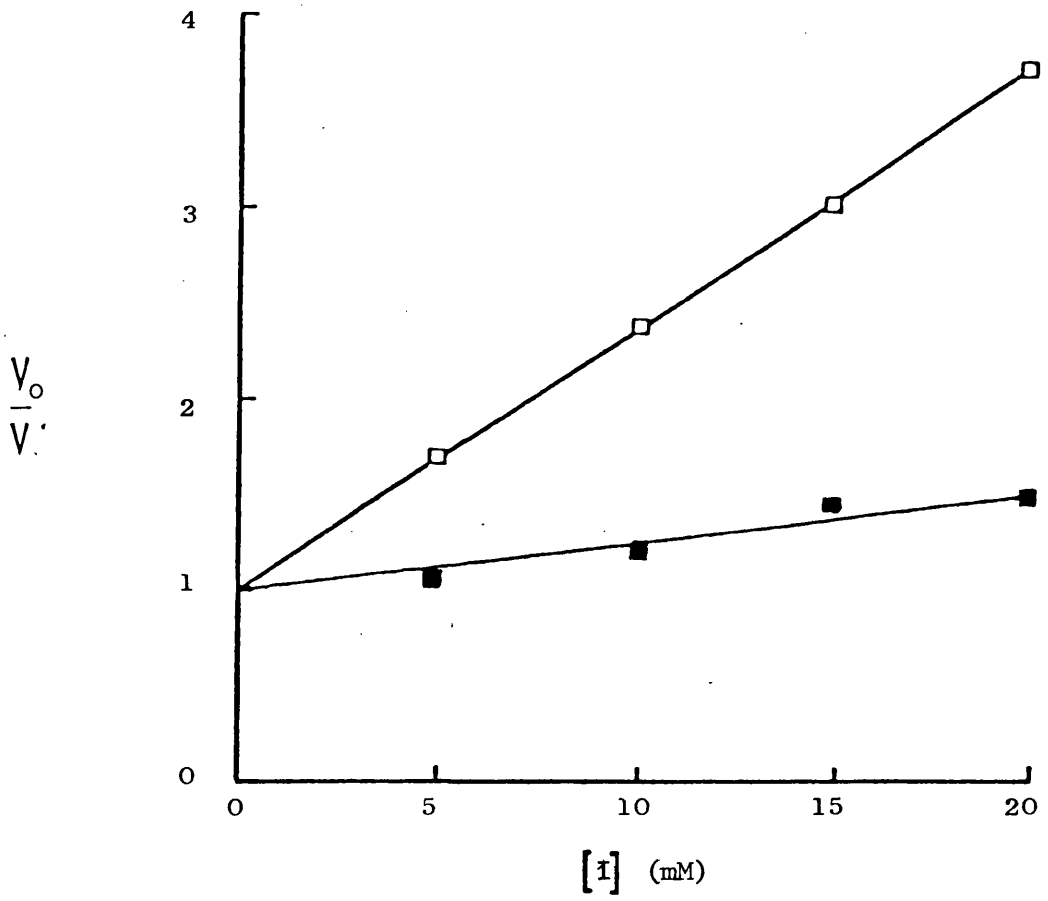
C-3 analogues

D-allose, the substrate used in these experiments is the C-3 epimer of D-glucose. It has a very low affinity (Km 271.3 ± 34.2mM) compared to D-glucose or D-galactose. Fig. 18 shows the inhibition of D-allose exchange by C-3 analogues of D-glucose. 3-deoxy-D-glucose also shows low affinity(Ki = 40.31 ± 4.21mM) indicating that a hydrogen bond is probably directed to the gluco-configuration hydroxyl at C-3. The presence of an allo configuration hydroxyl at C-3 interferes with binding. 3-deoxy-3-fluoro-D-glucose has a high affinity (Ki = 7.97 ± 0.44mM) suggesting that a hydrogen bond is probably directed towards the electronegative oxygen (or fluorine) at C-3, rather than from the hydrogen atom of the C-3 hydroxyl towards an electronegative group on the transporter.

C-4 and C-6 analogues

D-galactose, the C-4 epimer of D-glucose, has a lower affinity for the adipocyte hexose transporter than D-glucose. The Ki for D-galactose inhibition of D-allose exchange is 24.29 ± 3.05mM. If a hydrogen bond is directed towards a gluco configuration hydroxyl at the C-4 position of D-glucose during transport it is less important than those at the ring oxygen, C-1 and C-3. The possibility that the lowering of affinity is due either to a galacto-configuration hydroxyl interfering with binding at other positions, or to steric hindrance cannot be ex-

Fig 18.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-glucose analogues modified at C-3. (■) 3-deoxy-D-glucose, K_i = 40.3 ± 4.2 mM (S.E., n=10); (□) 3-deoxy-3-fluoro-D-glucose, K_i = 7.97 ± 0.44 mM (S.E., n=24).

cluded.

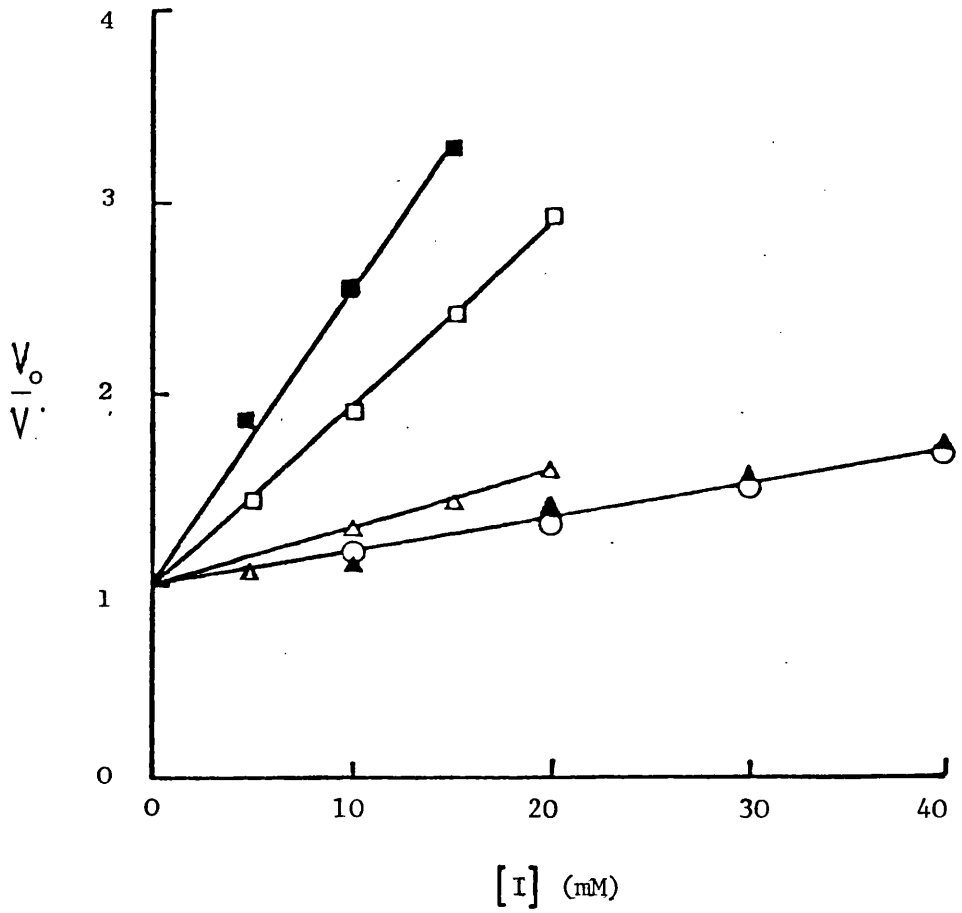
Fig. 19 shows the inhibition of D-allose exchange by C-4 and C-6 analogues of D-glucose. A C-6 hydroxyl or fluorine atom appears to slightly increase binding when these substitutions are compared with the relevant C-6 deoxy analogue. Thus 6-deoxy-D-glucose(Ki = 11.08 ± 0.63mM)has lower affinity than D-glucose, whilst D-fucose (6-deoxy-D-galactose Ki = 33.97 ± 5.03mM)has a lower affinity than D-galactose. 6-deoxy-6-fluoro-D-galactose has a Ki = 6.67 ± 0.37mM indicating a hydrogen bond is formed between the transporter and the C-6 hydroxyl but this bond is not as important as those bonds to the ring oxygen, C-1 and C-3. Removal of the C-6 hydroxyl results in a greater affinity loss for the D-galactose derivative (D-fucose) than for the corresponding D-glucose derivative (6-deoxy-D-glucose). This may indicate that the proposed hydrogen bond to C-6 becomes more important when the hydrogen bonding or spatial requirements at C-4 are not met.

Removing the C-6 carbon (C-5 hydroxy methyl) group altogether also reduces affinity. D-xylose can be regarded as a D-glucose analogue lacking the C-5 hydroxy methyl group and L-arabinose as a D-galactose analogue without the C-5 hydroxy methyl group. The Ki for D-xylose is 45.56 ± 3.25mM and for L-arabinose the Ki is 49.69 ± 4.93mM. Thus both analogues have low affinity with little discrimination between the D-glucose and D-galactose analogues.

D-xylose transport in adipocytes

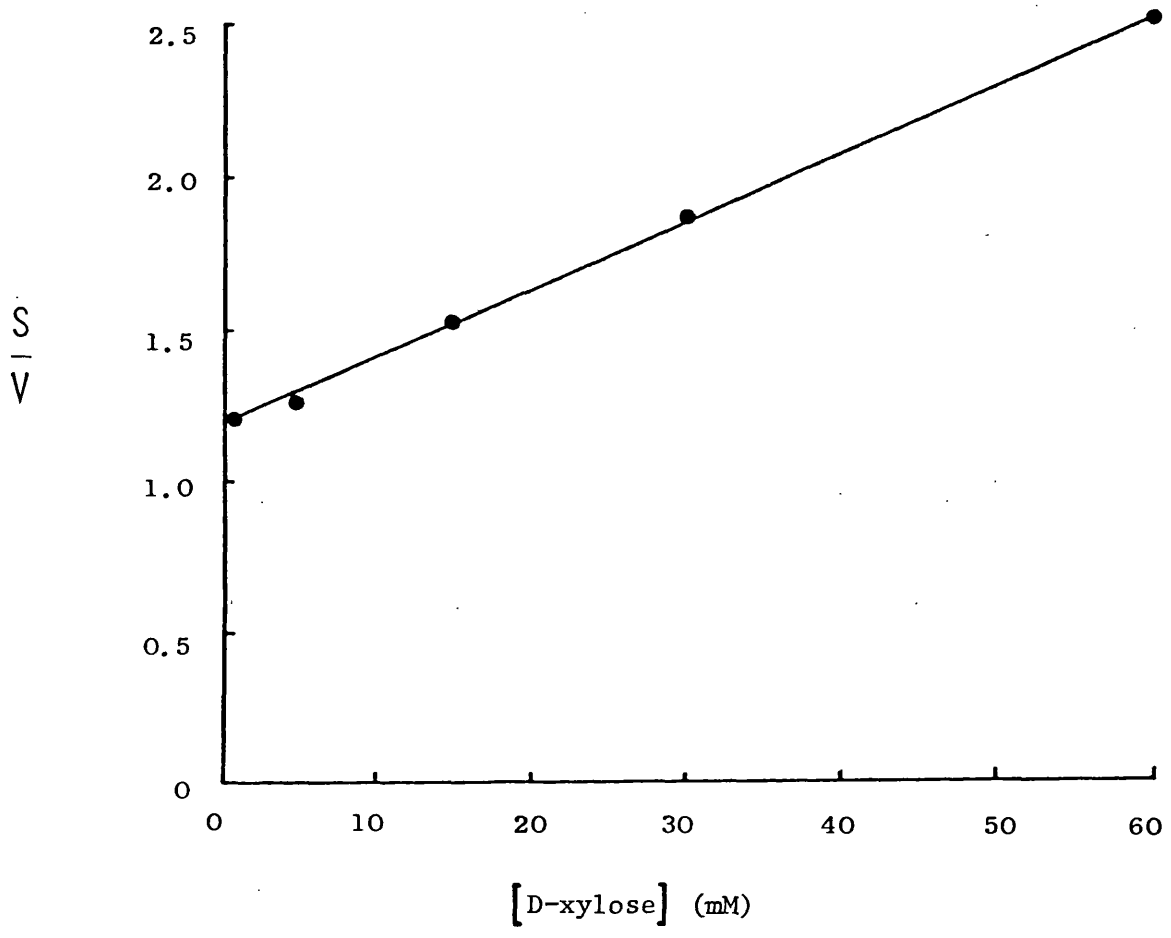
Fig. 20 shows the equilibrium exchange of D-xylose in insulin-stimulated adipocytes. The experiments were performed in an identical manner to the exchange of D-allose and 3-O-methyl-D-glucose using [U-^{14}C]-D-xylose as tracer. The D-xylose tracer was slowly metabolised by

Fig 19.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-glucose analogues modified at C-4 and C-6. (▲) L-arabinose, K_i = 49.69 ± 4.93 mM (S.E., n=21); (○) D-xylose, K_i = 45.56 ± 3.25 (S.E., n=24); (△) D-fucose, K_i = 33.97 ± 5.03 mM (S.E., n=15); (□) 6-deoxy-D-glucose, K_i = 11.08 ± 0.63 mM (S.E., n=11); (■) 6-deoxy-6-fluoro-D-galactose, K_i = 6.67 ± 0.37 mM (S.E., n=11).

Fig 20.



The equilibrium exchange of D-xylose in adipocytes treated with 10 nM insulin. K_{ee}^{oi} = 53.21 ± 1.45 mM, V_{ee}^{oi} = 0.75 ± 0.01 mM sec^{-1}. (Best fit estimates ± S.E. from weighted regression, n=5).

adipocytes since the D-xylose did not come to equilibrium and the radioactivity continued to be accumulated by the cells. The rate of metabolism of 1mM D-xylose was slow at 0.18nMol/h/10^5 cells (compared to 1.08nMol/h/10^5 cells for 0.12mM D-glucose) compared with the rate of transport, and therefore the equilibrium exchange parameters for D-xylose can be estimated. D-xylose shows a K_{ee}^{oi} = 53.21 ± 1.45mM and a V_{ee}^{oi} of 0.75 ± 0.01mM sec^{-1}. The Vmax is similar to that observed for the exchange of 3-O-methyl-D-glucose (see p.124) and D-allose (see p.121). The Km for D-xylose transport is similar to the Ki measured for D-xylose inhibition of D-allose exchange indicating that the Ki does provide a good measure of the apparent Km.

Spatial requirements for binding to the external site of the adipocyte hexose transporter

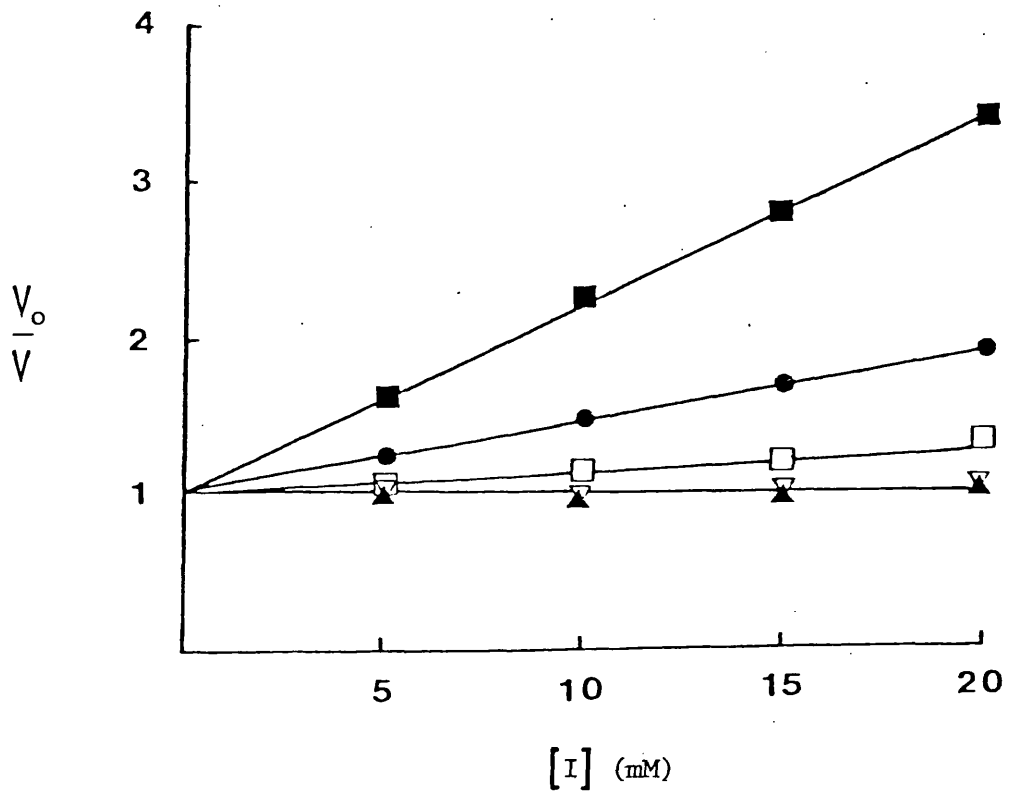
Alkyl substituted hexoses have previously been used to investigate the spatial requirements of the erythrocyte hexose transporter (Barnett et al., 1973b). In order to reveal the spatial features of the outside site some analogues were added with the substrate, but, where indicated, some analogues were allowed to equilibrate across the membrane for 30 minutes before the transport rate was estimated.

C-1 substitution

Fig. 21 shows the inhibition of D-allose exchange in insulin-treated adipocytes by a series of C-1 substituted D-glucose analogues. The inhibition is greatly reduced if a methyl group is introduced into the α or β configuration. Methyl-α-D-glucoside has a Ki of 57.9 ± 13.6mM while methyl-β-D-glucoside shows no detectable inhibition over the concentration range tested.

Also shown in Fig. 21 is a comparison of the affinities of two disaccharides. Maltose has a Ki = 23.5 ± 2.1mM whilst trehalose shows no detectable inhibition. This indicates that trehalose (α -1, 1, glucopyranosyl-glucopyranoside) is hindered from binding by the α-1, 1, linkage and the relatively unhindered C-4 and C-6 positions do not bind well. With maltose (α - 1, 1,4-glucopyranosyl-glucopyranose) there is an unhindered reducing glucose unit which is apparently able to bind to the external site with the bulky non-reducing glucose unit projecting from the site. This, together with the inability of the site to accept a methyl group at C-1, indicates that the external site requires a specific orientation of the sugar molecule and an unhindered C-1 position.

Fig 21.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by (△) β-methyl-D-glucoside (30 minute preincubation); (□) α-methyl-D-glucoside (30 minute preincubation), K_i = 57.9 ± 13.6 mM (S.E., n=19); (▲) trehalose; (●) maltose, K_i = 23.5 ± 2.1 mM (S.E., n=12); (■) D-glucose.

C-2 substituted analogues

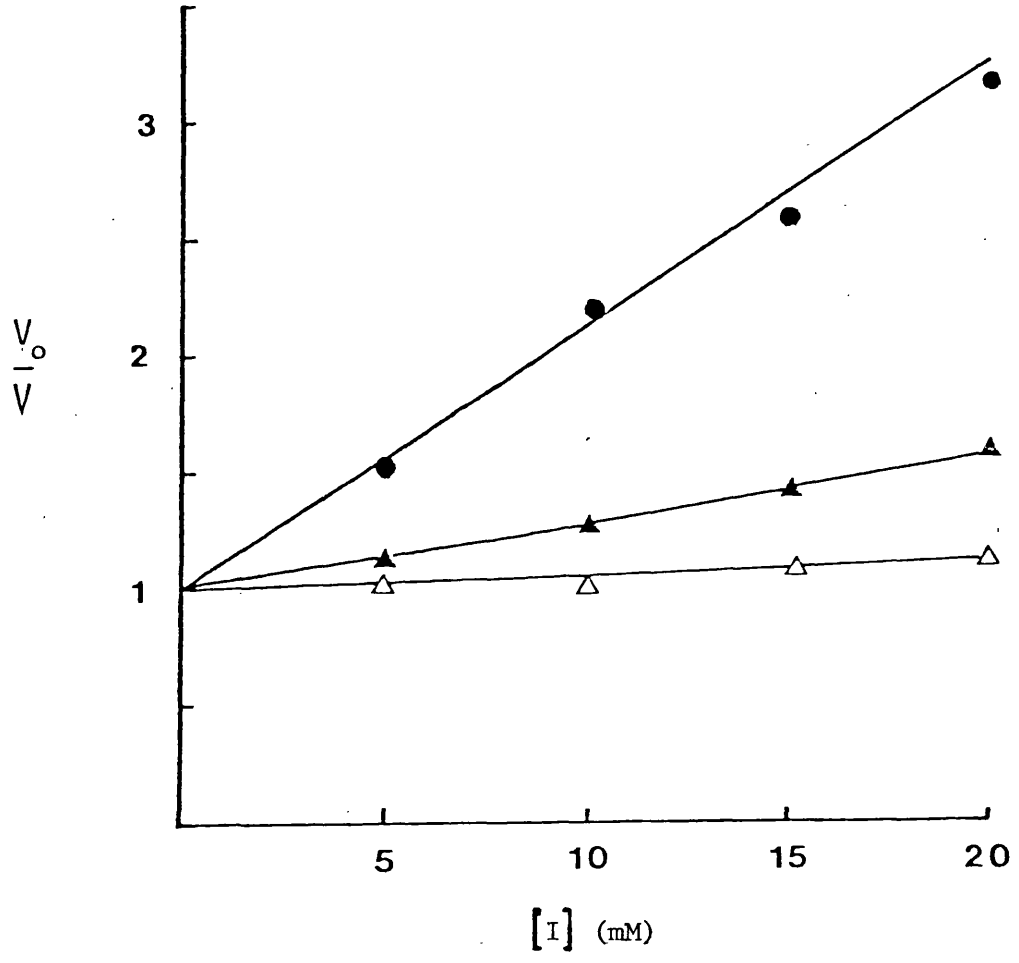
The results shown in Fig. 22 show the inhibition of D-allose exchange by C-2 substituted D-glucose analogues. D-glucosamine and N-acetyl-D-glucosamine were equilibrated with the cells for 30 minutes before the transport assay. D-glucosamine binds well (Ki = 9.05 ± 0.66mM) but the introduction of the acetate group in N-acetyl-D-glucosamine reduced the inhibition to undetectable levels.

If 3-O-methyl-D-glucose (Fig. 23) is compared with 2-3-di-O-methyl-D-glucose (Ki = 42.09 ± 7.51mM), then it is seen that the additional methyl group at C-2 markedly reduces affinity. This indicates that there is very little space around C-2 when the hexose molecule is bound to the transporter.

C-3 substituted analogues

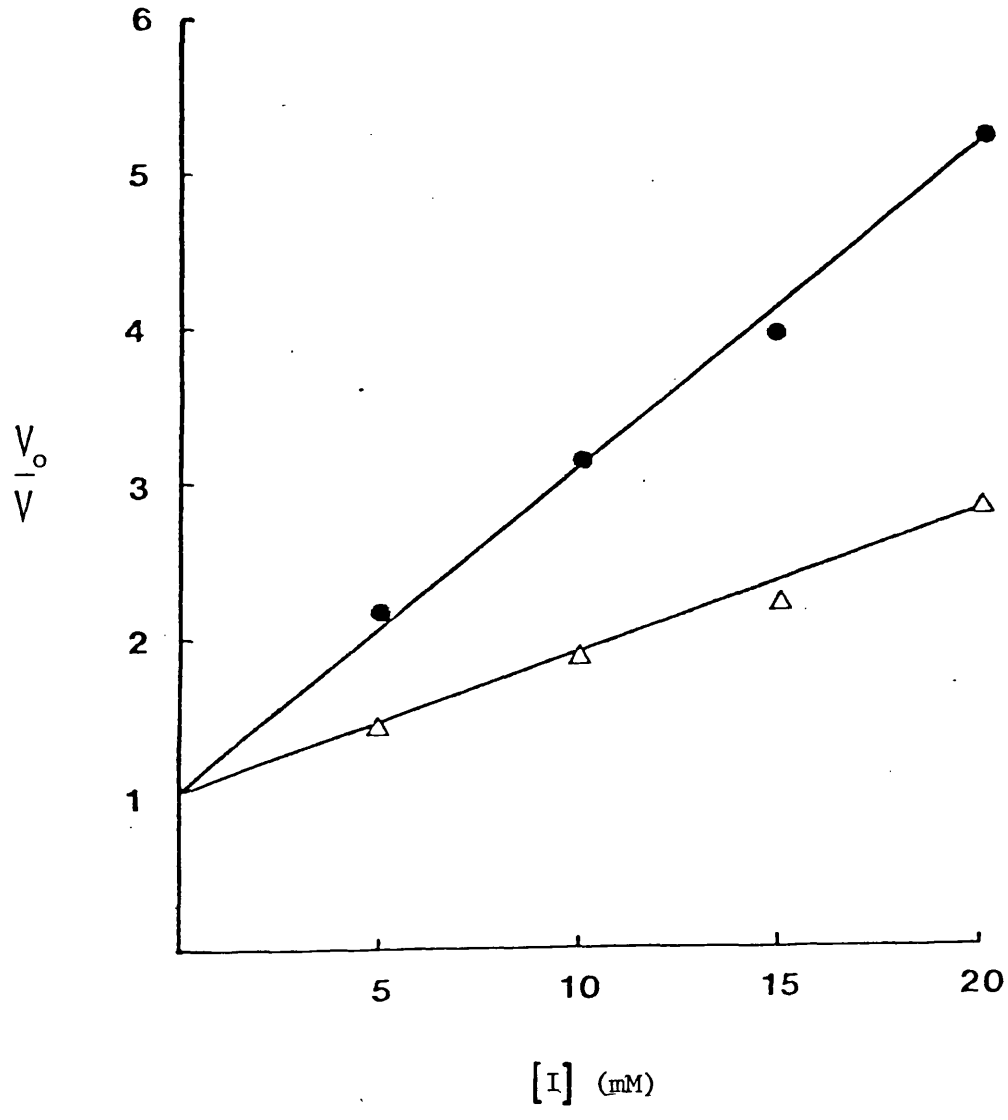
In contrast to the lack of space around C-1 and C-2, C-3 accepts a methyl group well (Fig. 23). 3-O-methyl-D-glucose has high affinity Ki 4.75 ± 0.36mM. The affinity is higher than D-glucose and may be due to hydrophobic bonding of the methyl group or, alternatively, the methyl group may confer increased electronegativity to the adjacent oxygen, which accepts a hydrogen bond from the transporter (see p. 132). The Ki calculated for 3-O-methyl-D-glucose inhibition of D-allose is very similar to the equilibrium exchange Km for 3-O-methyl-D-glucose (see p.124 also Taylor & Holman, 1981; Whitesell & Gliemann, 1979, and Vinten et al., 1976). Loten et al. (1976) also measured 3-O-methyl-D-glucose inhibition of D-allose uptake in adipocytes and reported a similar Ki value.

Fig 22.



The inhibition of D-allose transport (in cells treated with 10 nM insulin).(▲)2,3-di-O-methyl-D-glucose, K_i = 42.1 ± 7.5 mM (S.E., n=11); (●) D-glucosamine, K_i = 9.05 ± 0.66 mM (S.E., n=12); (△) N-acetyl-D-glucosamine. After 30 minute preincubation in each case.

Fig 23.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by (●) 3-O-methyl-D-glucose (30 minute preincubation) and by (△) 3-O-propyl-D-glucose, K_i = 11.26 ± 2.12 mM (S.E., n=12).

Increasing the size of the alkyl group at C-3 from a methyl to a propyl group results in a reduction of affinity. The Ki for 3-O-propyl-D-glucose when present only in the external solution is 11.26 ± 2.12mM. Thus, though there is more space around C-3 than at C-1 or C-2, the propyl group is subject to steric hindrance.

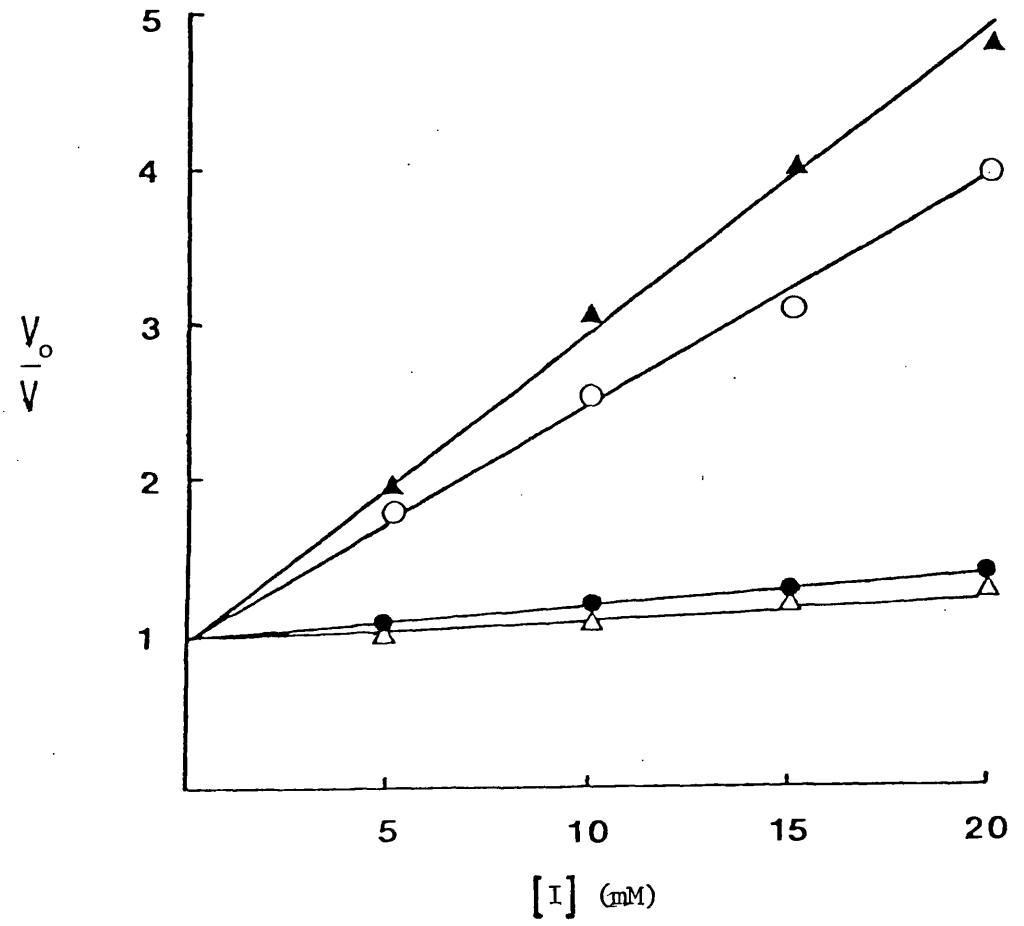
C-4 and C-6 substituted analogues

4,6-O-ethylidene-D-glucose is a good inhibitor of D-allose uptake (Fig. 24) with a Ki comparable with that of D-glucose. The Ki for 4,6-O-ethylidene-D-glucose is 6.11 ± 0.50mM. This result indicates that the bulky group directed towards C-4 does not interfere with binding, and supports the suggestion (based on the inhibition by maltose) that the sugar orientation in the outer transport site is such that C-4 projects into the external solution.

The results with C-6 substituted hexoses are less easy to interpret. 6-O-methyl-D-galactose and 6-O-propyl-D-glucose have low affinity compared to their parent hexoses. (The Ki for 6-O-methyl-D-galactose is 87.25 ± 17.85mM and for 6-O-propyl-D-glucose it is 78.1 ± 12.6mM). 6-O-pentyl-D-galactose is however a good inhibitor (Ki 4.66 ± 0.23mM) which may be due to hydrophobic interactions occurring. Some of this inhibition may be due to nonspecific or detergent-like action though no increased lysis was observed.

The difference between the high affinity of 4,6-O-ethylidene-D-glucose and the low affinity of 6-O-propyl-D-glucose (both compounds having a bulky substitution at C-6) is probably due to random orientation of the alkyl group that can occur in 6-O-propyl-D-glucose but not in 4,6-O-ethylidene-D-glucose. Thus the bulky group in 6-O-propyl-D-glucose could project towards and interfere with hydrogen bonding to the ring

Fig 24.



The inhibition of D-allose transport (in cells treated with 10 nM insulin) by (△) 6-O-methyl-D-galactose (30 minute preincubation) K_i = 87.3 ± 17.9 mM (S.E., n=9); (●) 6-O-propyl-D-glucose, K_i = 78.1 ± 12.6 mM (S.E., n=23); (▲) 6-O-pentyl-D-galactose, K_i = 4.66 ± 0.23mM (S.E., n=12); (○) 4,6-O-ethylidene-D-glucose, K_i = 6.11 ± 0.50 mM (S.E., n=12).

oxygen, while in 4,6-O-ethylidene-D-glucose the bulky group must project towards C-4.

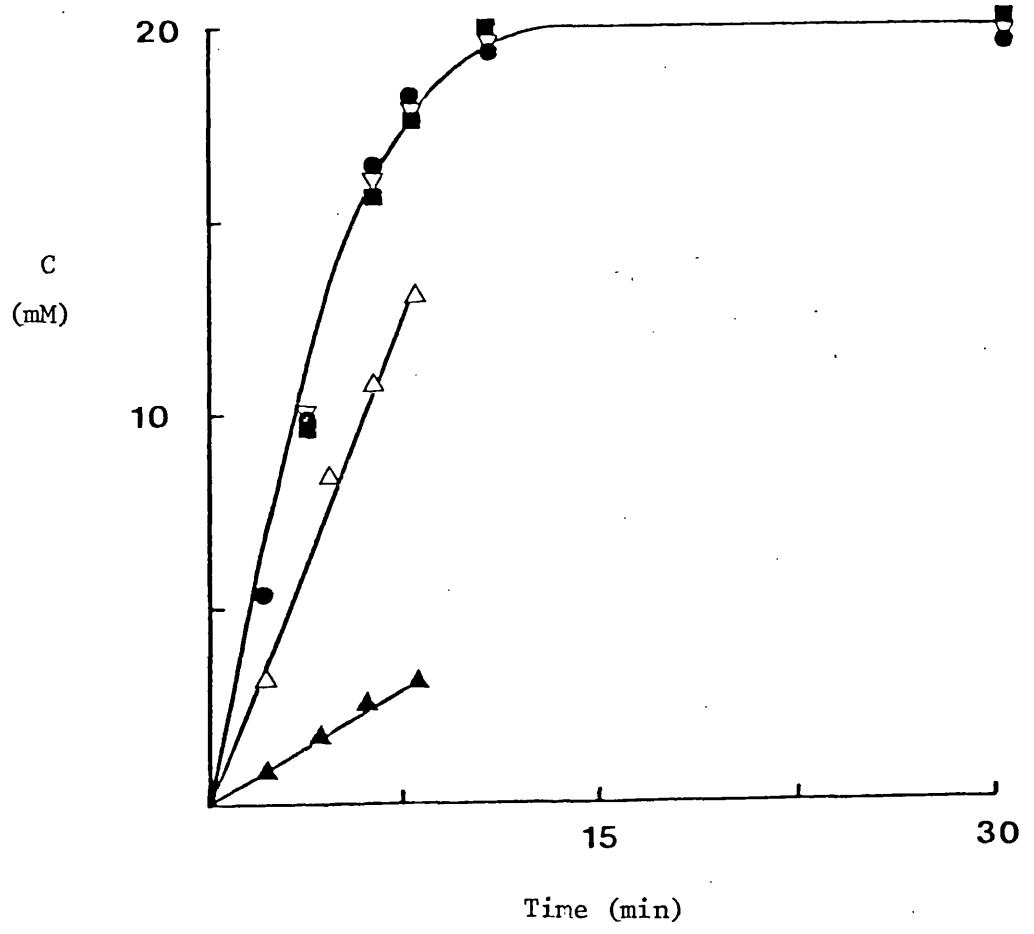
Side specific analogues for the adipocyte hexose transport system

Baker & Widdas (1973a) using an osmotic swelling method, have shown that 4,6-O-ethylidene-D-glucose is not transported by the human erythrocyte hexose transport system but enters the cell by an alternative route that is D-glucose insensitive.

Fig. 25 shows the uptake of 20mM 4,6-O-ethylidene-D-[U-^{14}C]glucose by an insulin-stimulated adipocyte preparation at 37°C and 16°C. At 37°C this compound equilibrates across the adipocyte plasma membrane in less than 15 minutes (the rate constant is 0.228 min^{-1}). The rate of 16°C is slower with a rate constant of 0.0213 min^{-1}. Fig. 31 also shows that the uptake of 4,6-O-ethylidene-D-glucose is inhibited by 0.3mM phloretin (rate constant 0.124 min^{-1}).

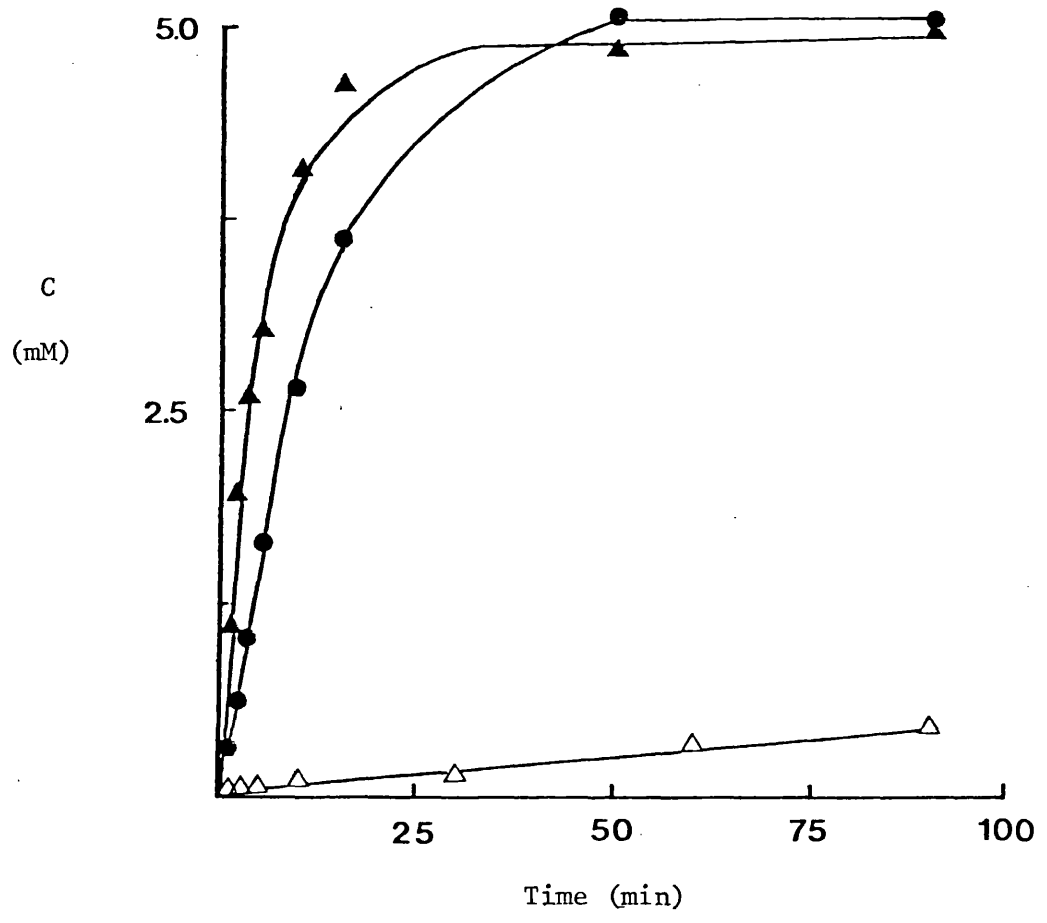
Barnett et al. (1973b, 1975) reported that n'-propyl-β-D-glucoside also penetrates the erythrocyte plasma membrane independently of the hexose transport system. Fig. 26 shows the uptake of 5mM n'-butyl-β-D-[6-^{3}H]-glucoside, 5mM n'propyl-β-D-[6-^{3}H]-glucoside and 5mM methyl-β-D-[6-^{3}H]-glucoside by adipocytes at 37°C. The 4,6-O-ethylidene-D-glucose, n'-propyl-β-D-glucoside and n'-butyl-β-D-glucoside space at equilibrium was identical to the space occupied by 3-O-methyl-D-glucose at equilibrium and equal to 1.8µl/100µl packed cells. This value remained constant for at least 90 min for all four sugars. The methyl-β-D-glucoside did not reach equilibrium over the time course studied and the rates are calculated for uptake into the 3-O-methyl-D-glucose space measured in the

Fig 25.



Uptake of 20mM 4,6-O-ethylidene-[U-^{14}C]-D-glucose at 37°C in basal adipocytes (▽); in adipocytes treated with 10 nM insulin (●); and in insulin treated cells in the presence of 50mM D-glucose (■). Also shown is 4,6-O-ethylidene-D-glucose uptake in the presence of 0.3 mM phloretin (△) and uptake at 16°C(▲).(2 experiments with duplicate observations except phloretin and low temperature treatment which were single experiments in duplicate).

Fig 26.



The uptake of 5 mM methyl-β-D-glucoside (△); n'propyl-β-D-glucoside (●); and n'butyl-β-D-glucoside (▲) in insulin treated cells at 37°C. Single experiments with triplicate observations in each case).

same experiment. These results indicate that there is no breakdown or metabolism of these compounds over a 90 min incubation time.

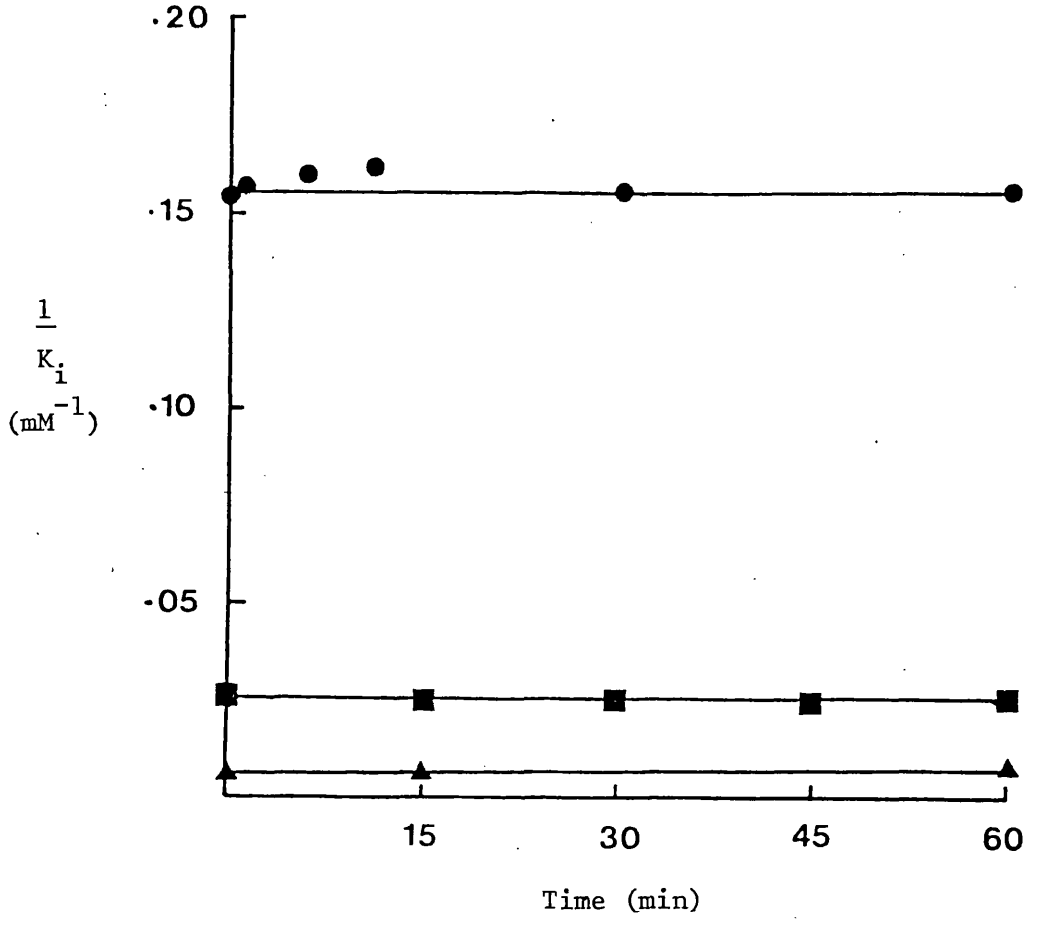
Table 9 summarises the rate constants for the uptake of 4,6-O-ethylidene-D-glucose and the n'alkyl-β-D-glucosides measured in the presence and absence of insulin, and shows that these compounds are taken up by a route insensitive to insulin. Table 9 also shows that the uptake of these compounds is not inhibited by 50mM D-glucose. The uptake rates are considerably increased by increasing the size of the alkyl group and this is consistent with penetration through lipid regions of the membrane. The uptake rates increase (Table 9) in the order methyl < propyl < butyl < ethylidene.

4,6-O-ethylidene-D-glucose added to the external solution of a rat adipocyte suspension inhibits the exchange of D-allose with a Ki = 6.11mM (see p. 143). The Ki for the inhibition of 40μM 3-O-methyl-D-glucose exchange by 4,6-O-ethylidene-D-glucose added to the external solution is 6.54 ± 0.56mM and is thus similar to the Ki estimated using D-allose as the substrate. The exchange of 40μM 3-O-methyl-D-glucose is rapid ($t_{\frac{1}{2}}$ = 3.15 sec) and exchange was measured over a period of three seconds. Thus estimates of the inhibition of 3-O-methyl-D-glucose exchange can be made rapidly before the inhibitor has significantly penetrated the membrane. Additional inhibition by 4,6-O-ethylidene-D-glucose as it reaches the internal solution of the adipocyte would be expected to reduce the Ki for 3-O-methyl-D-glucose exchange. Fig. 27 shows that the Ki for 4,6-O-ethylidene-D-glucose remains constant for up to 90 minutes indicating that there is no increase in affinity for this compound as it reaches the inside site of the sugar transport system.

Table 9. Uptake of glucose analogues

	Basal cells (rate constant min^{-1})	+ 10nM insulin (rate constant min^{-1})	+ 10nM insulin + 50mM D-glucose (rate constant min^{-1})
4,6-O-ethylidene-D-glucose	0.221 ± 0.027 (n = 3)	0.222 ± 0.038 (n = 4)	0.202 ± 0.035 (n = 3)
methyl-β-D-glucoside	-	0.0035 ± 0.0004 (n = 10)	-
n'-propyl-β-D-glucoside	0.061 ± 0.002 (n = 6)	0.076 ± 0.003 (n = 15)	0.061 ± 0.004 (n = 6)
n'-butyl-β-D-glucoside	0.202 ± 0.015 (n = 6)	0.238 ± 0.015 (n = 12)	0.197 ± 0.007 (n = 6)

Fig 27.



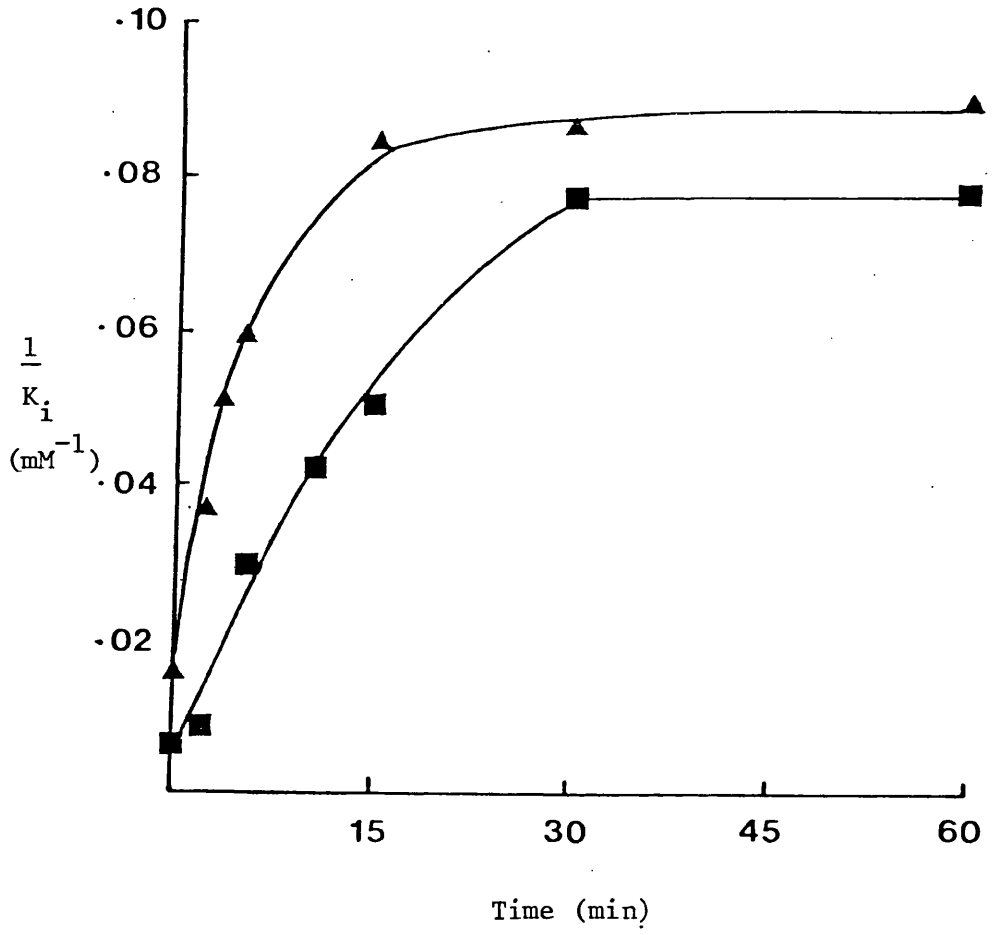
A plot of $1/K_i$ against time of incubation with 20 mM inhibitor. (●) 4,6-O-ethylidene-D-glucose; (■) 6-O-propyl-D-galactose; and (▲) methyl-β-D-glucoside. (2-3 experiments with triplicate observations in each case). Cells were insulin treated and at 37°C

Also shown in Fig. 27 is $^{1}/_{Ki}$ for 6-O-propyl-D-galactose plotted against increasing preincubation time. The Ki for 6-O-propyl-D-galactose is high and, within experimental error, is approximately constant over the course of the experiment.

Fig. 28 shows the inhibition of 40μM 3-O-methyl-D-glucose exchange by n'-propyl-β-D-glucoside and by n'-butyl-D-glucoside with increasing preincubation time. As the glucosides enter the intracellular space the Ki for n'butyl-β-D-glucoside changes from 63.25 ± 8.85mM to 10.98 ± 1.23mM while the Ki for n'-propyl-β-D-glucoside changes from 138.3 ± 47.5mM to 13.4 ± 2.2mM in the same period. There is no change in the final inhibition constants for longer preincubation times up to 90 minutes. Methyl-β-D-glucoside shows no change in Ki over a 90 minute period which is consistent with the very slow penetration rate for this compound. Thus in each case the Ki closely follows the penetration rates for the glucosides, indicating that C-1 alkyl glucosides can inhibit hexose transport when in the internal solution. Fig. 29 shows the inhibition of 40μM 3-O-methyl-D-glucose exchange by phenyl-β-D-glucoside with increasing incubation time. The rapid penetration of phenyl-β-D-glucoside and n'butyl-β-D-glucoside may account for the apparent external inhibition shown by these compounds, since the inhibitor may be able to enter the cell during the assay period and give detectable inhibition at the inner site.

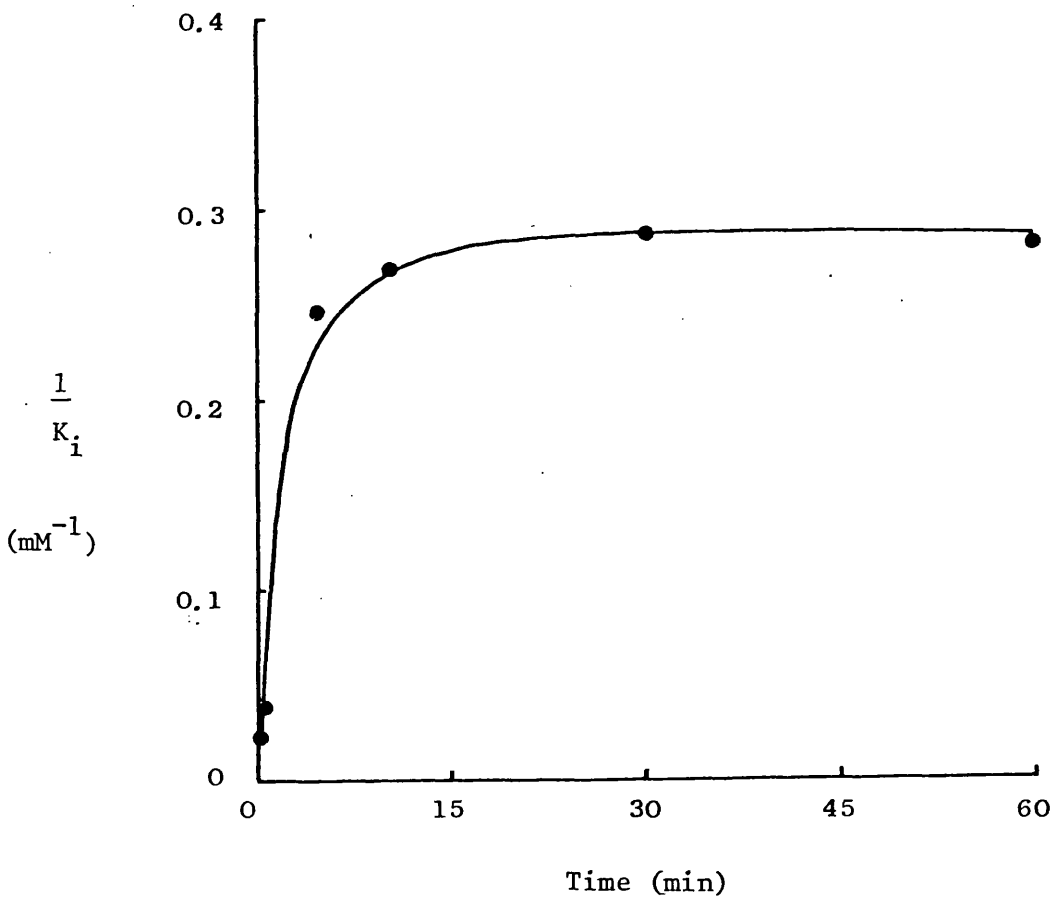
Fig. 30 shows the inhibition of 1.3mM D-allose exchange by n'-propyl-β-D-glucoside after a 30 minute incubation. This time is sufficient to equilibrate the alkyl sugar to equal concentrations on both sides of the membrane. The Ki for n'-propyl-β-D-glucoside inhibition of D-allose exchange is 17.98 ± 1.46mM. In the same experiments, cells were equilibrated with 20mM n'-propyl-β-D-glucoside for 30 minutes, then

Fig 28.



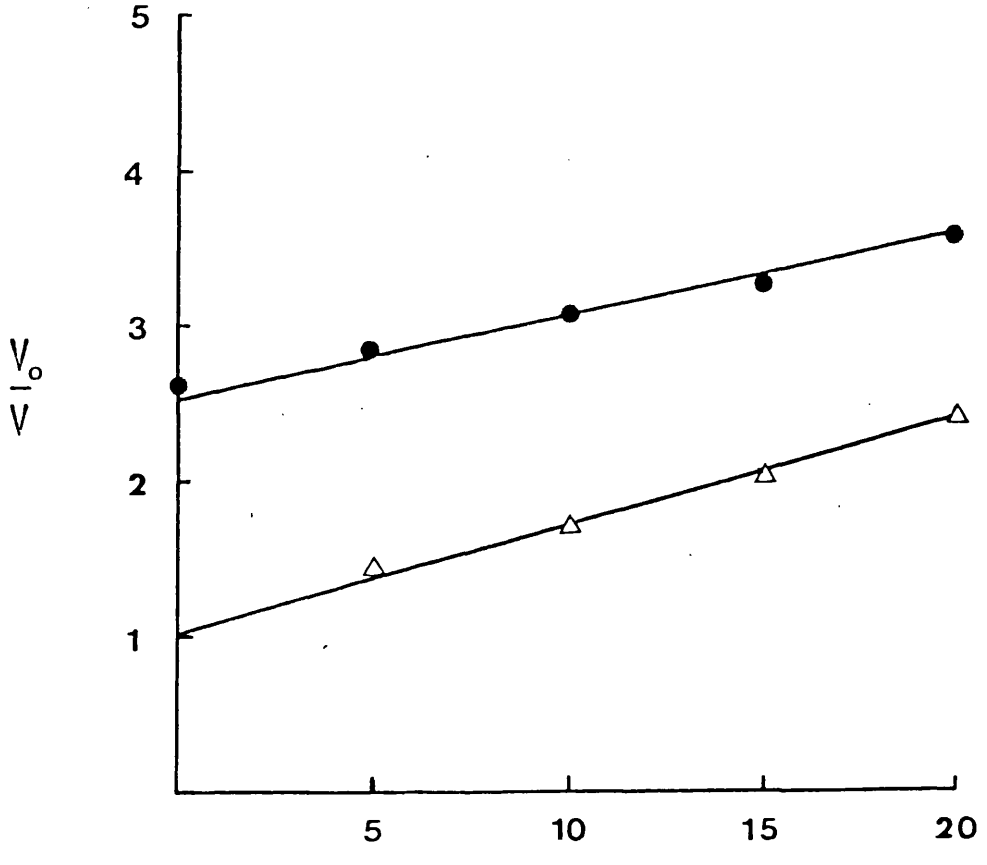
A plot of $1/K_i$ against time of incubation with 20 mM inhibitor. (■) n'propyl-β-D-glucoside and (▲) n'butyl-β-D-glucoside. (2 experiments with triplicate observations in each case). Cells were insulin treated and at 37°C in each case.

Fig 29.



A plot of $1/K_i$ against time of incubation with 20 mM phenyl-β-D-glucoside. (2 experiments with triplicate observations). Cells were insulin treated and at 37°C.

Fig 30.



The inhibition of 1.3 mM D-allose uptake in insulin treated cells at 37°C by n'propyl-β-D-glucoside (K_i = 17.98 ± 1.46 (S.E., n=30)) (△); and by n'propyl-β-D-glucoside in the presence of 5 mM 3-O-methyl-D-glucose (●). (K_i^{app} = 50.68 ± 6.26 (S.E., n=15)).

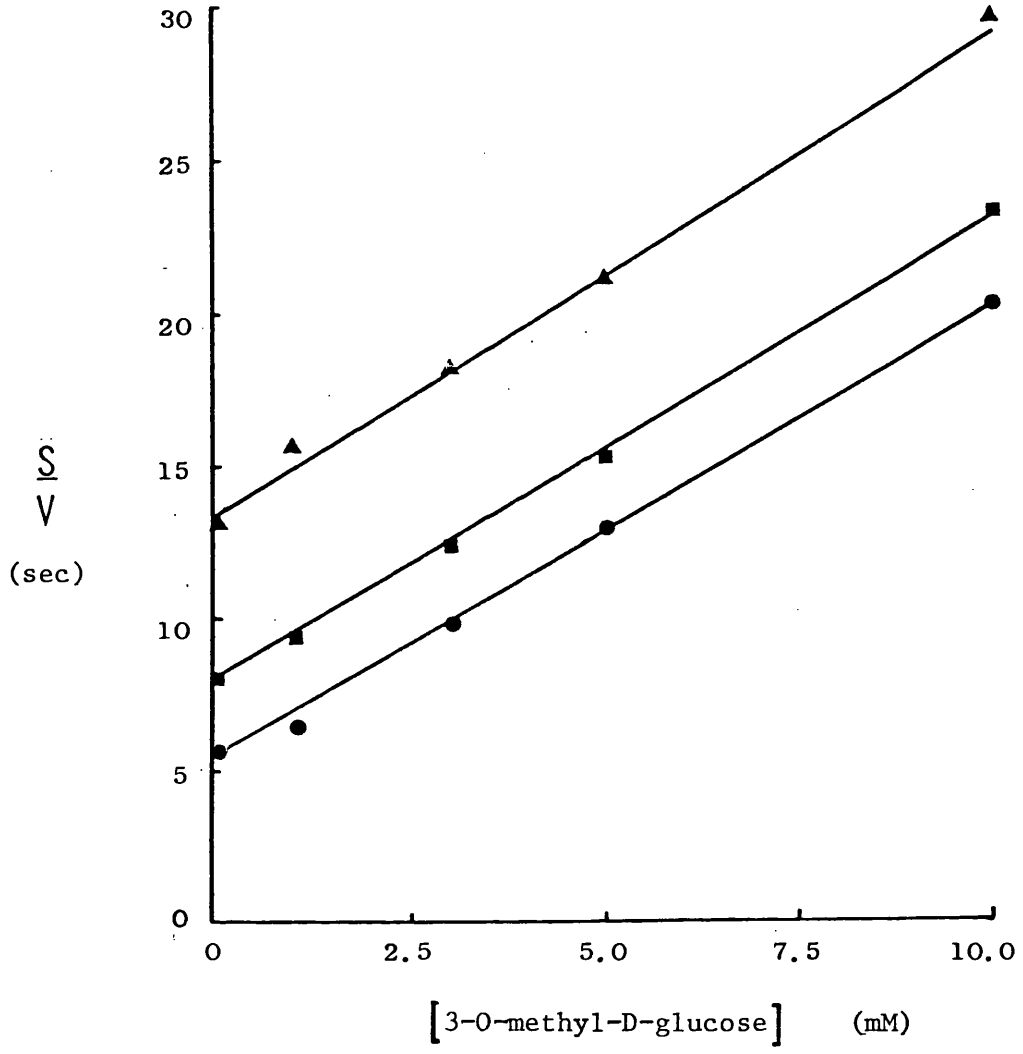
washed twice over a period of 30 minutes in 1% albumin-Hepes buffer at 37°C before the transport assay was performed. The cells showed no detectable inhibition of D-allose exchange, indicating that n'-propyl-β-D-glucoside inhibition is reversible and that no permanent effect on the cells occurs. The inhibition of D-allose exchange by 20mM ethanol and by 20mM propan-1-ol after 30 minutes preincubation is slight with the ethanol Ki > 100mM and the propan-1-ol Ki ≈ 150mM.

Also shown in Fig. 30 is the inhibition of D-allose exchange by n'-propyl-β-D-glucoside in the presence of 5mM 3-O-methyl-D-glucose with both sugars equilibrated across the membrane. 3-O-methyl-D-glucose increases the Ki for n'-propyl-β-D-glucoside indicating that the glucoside is displaced from its binding site by 3-O-methyl-D-glucose.

Fig. 31 shows the inhibition of 3-O-methyl-D-glucose exchange by 10mM 4,6-O-ethylidene-D-glucose and 10mM n'-propyl-β-D-glucoside. In both cases the analogues show competitive inhibition of 3-O-methyl-D-glucose exchange. This result indicates that both analogues are competitive with transported sugars with respect to their binding sites.

In order to confirm that the alkyl-β-D-glucosides are inhibitors at the inner face of the plasma membrane, zero-trans exit experiments were performed. In zero trans exit experiments the inhibitor is only present at the inner face of the plasma membrane, inhibitor present on the outside being diluted by 100-fold. Thus inhibitors acting at the outside binding site would not be expected to inhibit 3-O-methyl-D-glucose exit. Table 10 shows the Ki's calculated for the inhibition of 40μM 3-O-methyl-D-glucose exit at 37°C. 20mM n'-propyl-β-D-glucoside is an effective inhibitor whereas 20mM 4,6-O-ethylidene-D-glucose and 6-O-propyl-D-galactose are not. The determined Ki's are subject to a large error due

Fig 31.



The equilibrium exchange of 3-O-methyl-D-glucose in insulin treated adipocytes (●), K_m^{app}=3.61 ± 0.19mM,and in the presence of 10mM n'-propyl-β-D-glucoside (■), K_m^{app}=5.21 ± 0.14mM,and 10mM 4,6-O-ethylidene-D-glucose (▲), K_m^{app}= 8.32 ± 0.52 mM . (Best fit estimates ± S.E. from weighted regression, n=5 in each case).

Table 10. Inhibition of 3-O-methyl-D-glucose tracer exit by alkyl sugar derivatives inside the cell

Inhibitor		Ki (mM)	
20mM	3-O-methyl-D-glucose	6.42 ± 2.34	(n = 6)
(corrected for efflux using equation 11)		5.65 ± 2.05	(n = 6)
20mM	4,6-O-ethylidene-D-glucose	143.2 ±48.9	(n = 12)
20mM	n'-propyl-β-D-glucoside	8.88 ± 1.64	(n = 11)
20mM	6-O-propyl-galactose	433.9 ±87.9	(n = 12)

Average tracer efflux $t_{\frac{1}{2}}$ = 3.48 ± 0.42 sec (n = 12)

to the technical difficulties involved in measuring zero trans exit in adipocytes, but clearly show that the inner site accepts n'-propyl-β-D-glucoside but not 4,6-O-ethylidene-D-glucose. The lack of detectable inhibition in this experiment by 6-O-propyl-D-galactose indicates that C-6 alkylated sugars may not bind well to the inside site. Taken with the 3-O-methyl-D-glucose exchange results, this indicates that 6-O-propyl-D-galactose may be a better external inhibitor than internal inhibitor but the Ki's for both sites are high and no great confidence in the Ki estimates for this compound can be claimed.

6-O-propyl-D-galactose is thus a poor side specific analogue but 4,6-O-ethylidene-D-glucose and n'-propyl-β-D-glucose are very side specific for the external and internal sites of the transporter respectively.

A comparison of the hydrogen bonding and spatial requirements for binding to the adipocyte hexose transporter in basal and insulin treated cells

Table 11 compares the spatial and hydrogen bonding requirements of insulin-treated and basal cells. 3-O-methyl-D-glucose was used as the substrate in basal cells. The 3-O-methyl-D-glucose/D-allose permeability ratio is the same in both basal and insulin-treated cells. Also the similarity of the Km for 3-O-methyl-D-glucose transport and the Ki for 3-O-methyl-D-glucose inhibition of D-allose exchange suggests that the two sugars share the same transport system. Thus a comparison of the Ki values in basal and insulin-treated cells using two different substrates is reasonable.

Table 11. A comparison of the sugar transport specificity in basal and insulin-treated cells at 37°C

In basal cells 40μM 3-O-methyl-D-glucose was the substrate and Ki values were determined at 20mM inhibitor. The mean basal uninhibited uptake rate is $t_{\frac{1}{2}}$ = 3.14 ± 0.5 min which is about 55 times slower than in insulin-treated cells. The Ki values are compared with the present and previously reported results on insulin-treated cells using D-allose as the substrate.*

Inhibitor	Basal Ki (mM)	Insulin (10nM) Ki (mM)
D-glucose	9.61 ± 2.24 (n=12)	8.62 ± 0.71 (n=34)
5-thio-D-glucose	37.58 ± 6.6 (n=5)	42.1 ± 6.0 (n=12)
1-deoxy-D-glucose	NDI (n=6)	NDI (n=12)
methyl-β-D-glucoside	NDI (n=6)	NDI (n=12)
2-deoxy-D-galactose	21.58 ± 2.04 (n=6)	20.75 ± 3.04 (n=9)
2-3-di-O-methyl-D-glucose	38.73 ± 5.54 (n=6)	42.09 ± 7.48 (n=11)
3-O-propyl-D-glucose	8.93 ± 0.8 (n=6)	11.26 ± 2.12 (n=12)
3-deoxy-D-glucose	32.53 ± 1.58 (n=3)	40.31 ± 4.20 (n=10)
4-6-O-ethylidene-D-glucose	8.00 ± 3.53 (n=6)	6.11 ± 0.50 (n=12)
D-galactose	30.50 ± 7.22 (n=6)	24.49 ± 3.05 (n=12)
D-fucose	35.34 ± 8.59 (n=4)	39.35 ± 5.03 (n=15)
6-deoxy-D-glucose	14.17 ± 3.91 (n=6)	11.08 ± 0.63 (n=11)
6-O-methyl-D-galactose	98.31 ±30.18 (n=6)	87.25 ±17.85 (n=9)
6-O-pentyl-D-galactose	5.11 ± 1.51 (n=5)	4.66 ± 0.23 (n=12)

Results are mean ± S.E.M.

* The ratio of tracer permeability of 3-O-methyl-D-glucose/D-allose is approximately equal to 45 for basal and insulin treated cells.

In basal cells the poor hydrogen bonding analogues 5-thio-D-glucose, 1-deoxy-D-glucose, 3-deoxy-D-glucose, D-fucose and 6-deoxy-D-glucose indicate that as in insulin treated cells the important hydrogen-bonding positions are the ring oxygen, C-1, and C-3 with a less important hydrogen bonding group at C-6. The low affinity of methyl-β-D-glucoside, 2,3-di-O-methyl-D-glucose, 3-O-propyl-D-glucose and 6-O-methyl-D-galactose for the hexose transport system of basal cells indicates that insulin has no effect on the spatial requirements of the site. Table 11 also shows that D-glucose, D-galactose, 4,6-O-ethylidene-D-glucose and 6-O-pentyl-D-galactose have similar affinities in basal and insulin-treated cells. It can thus be concluded that insulin does not cause a major shape change in the site which might have been expected to affect the specificity requirements. Instead insulin probably leads to an increase in the number of sites available, with identical specificity.

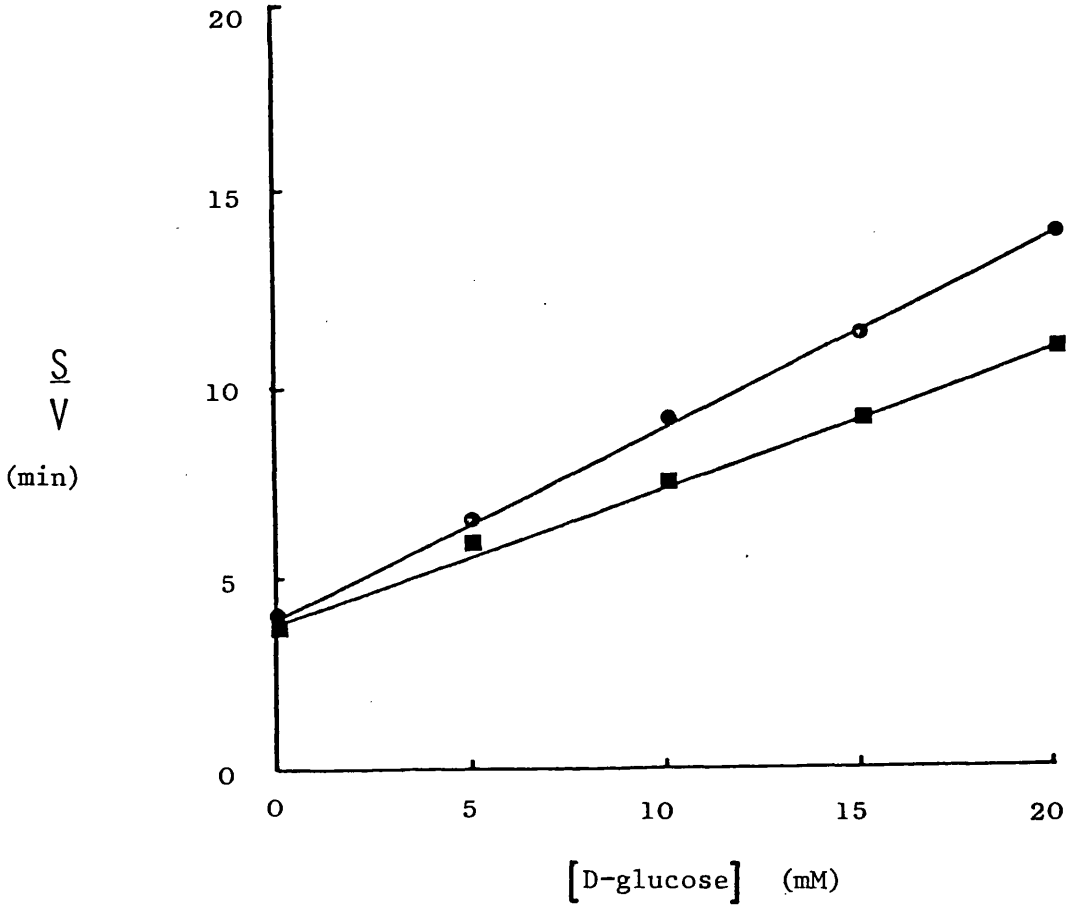
The effect of metabolism on the regulation of hexose transport

Foley et al. (1980a) reported that sugar metabolites accelerated the transport of hexoses in the adipocyte. In order to further investigate the role of metabolites, the inhibition of D-allose transport by metabolised and non-metabolised D-glucose analogues was studied.

Fig. 32 shows the inhibition of tracer D-allose exchange by D-glucose in the presence and absence of 2mM potassium cyanide. In normal cells these results show D-glucose to have a Ki of 8.62 ± 0.71mM for the inhibition of D-allose transport. Loten et al. (1976) reported a Ki of 14.8mM for the same experiment. Other groups however have reported a lower Ki value. Whitesell & Gliemann (1979) have reported a D-glucose Ki of 7mM using tracer 3-O-methyl-D-glucose as a substrate while Foley et al. (1978) using L-arabinose as substrate, reported a Ki for D-glucose of 8mM.

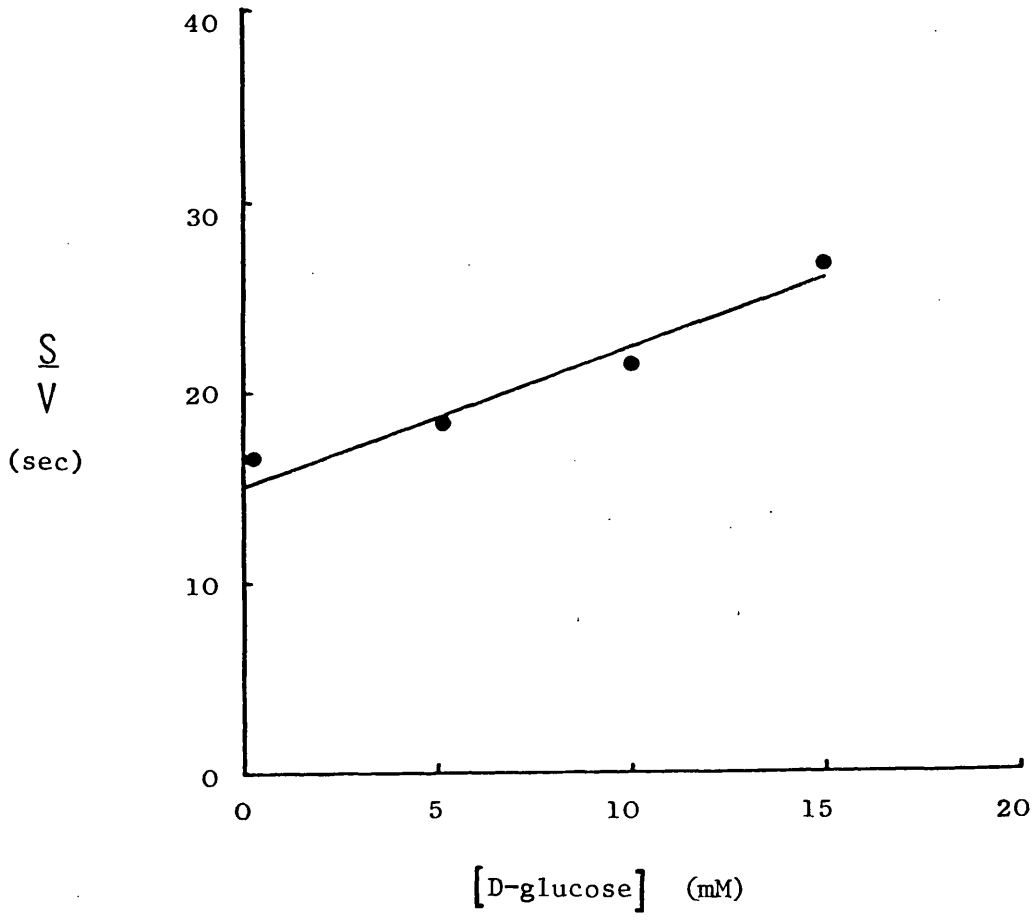
Isolated plasma membranes behave differently. Fig. 33 shows the S/V vs S plot for zero trans entry of D-glucose in plasma membranes prepared from insulin-treated adipocytes. This shows the plasma membrane system to have a Km of 23.9 ± 3.9 mM (n = 4) at 37°C. Ludvigsen & Jarett (1980) reported a D-glucose Km of 9-26mM for D-glucose transport in plasma membranes isolated from insulin-stimulated adipocytes. The change in Km was found to be related to the incubation time prior to homogenisation. The Km increased as the preincubation was extended. In the experiments presented here, the cells were preincubated for 15 min before homogenisation. Further evidence for a difference in kinetic parameters between whole cells and isolated membranes is provided by the results of Cushman & Wardzala (1980) who reported that the Ki for D-glucose inhibition of cytochalasin B binding to isolated plasma membranes

Fig 32.



The inhibition of D-allose exchange (in cells treated with 10 nM insulin) by D-glucose in the absence of potassium cyanide (●), K_i = 8.62 ± 0.71 mM (S.E., n=34); and in cells treated with 2mM potassium cyanide (■), K_i = 12.08 ± 0.68 mM (S.E., n=12).

Fig 33.



The equilibrium exchange of D-glucose in plasma membranes from insulin treated cells. K_m = 23.9 ± 3.9 mM and V_{max} = 1.65 ± 0.46 mM sec^{-1}. (Best fit estimates ± S.E., n=4).

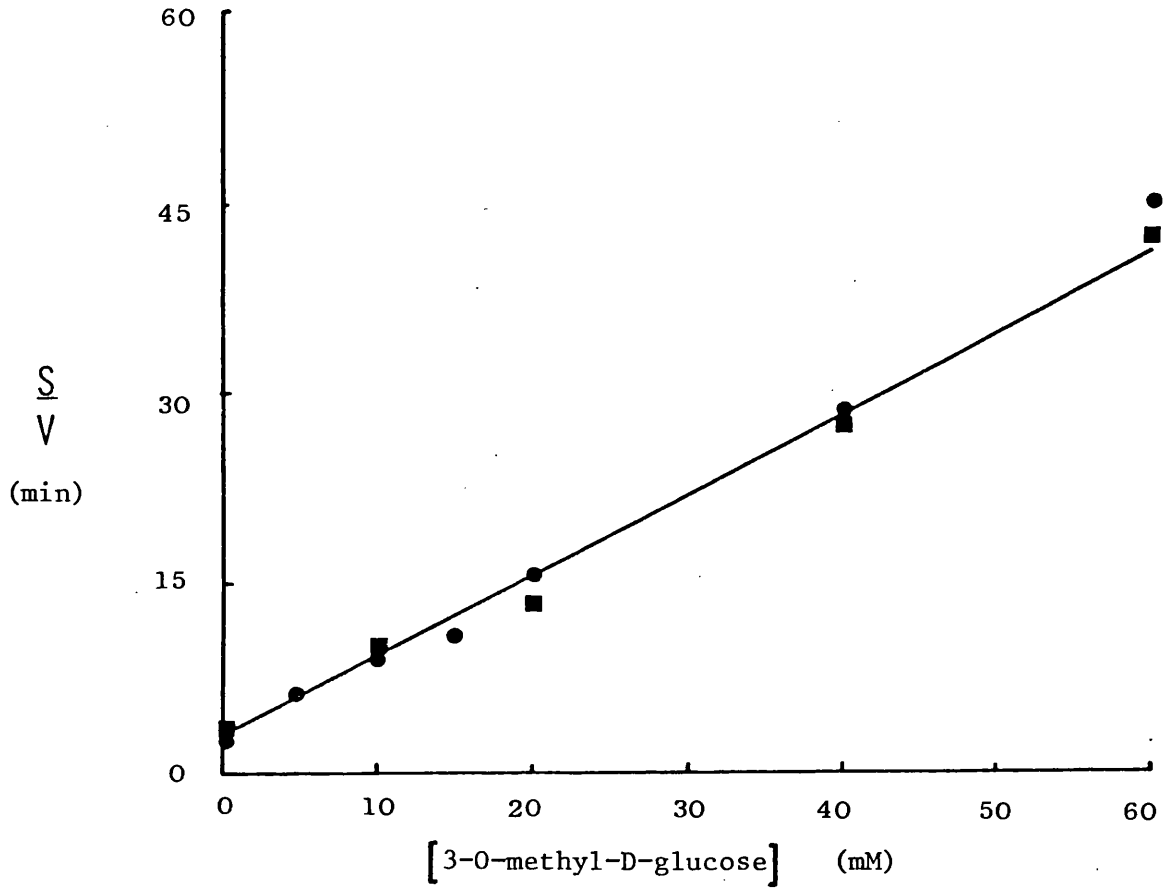
was 30mM.

One possible cause of this anomaly may be the regulation of transport in intact adipocytes by sugar metabolites. In order to investigate the transport process separately from metabolism the adipocytes were treated with 2mM potassium cyanide after treatment with insulin. This concentration of cyanide rapidly depletes the intracellular ATP levels (Kono et al., 1976, 1977) and thus prevents the phosphorylation of hexoses by hexokinase. Fig. 32 shows the effect of potassium cyanide treatment on the inhibition of D-allose exchange by D-glucose. The Ki for D-glucose changes from 8.62 ± 0.71mM in control cells to 12.08 ± 0.68mM in cyanide-treated cells. The presence of 2mM potassium cyanide does not affect either the D-allose equilibrium volume or the rate of tracer D-allose exchange.

Fig. 34 shows the inhibition of D-allose exchange by 3-O-methyl-D-glucose in adipocytes in the presence and absence of cyanide. There is no significant change in the Ki of 3-O-methyl-D-glucose, the Ki for control cells is 4.75 ± 0.36mM (n = 18) whilst in cyanide-treated cells it is 4.77 ± 0.48mM (n = 12). This confirms the observations with D-allose and is consistent with the observations of Kono et al. (1977) who showed no effect of cyanide poisoning on the rate of 3-O-methyl-D-glucose transport in adipocytes.

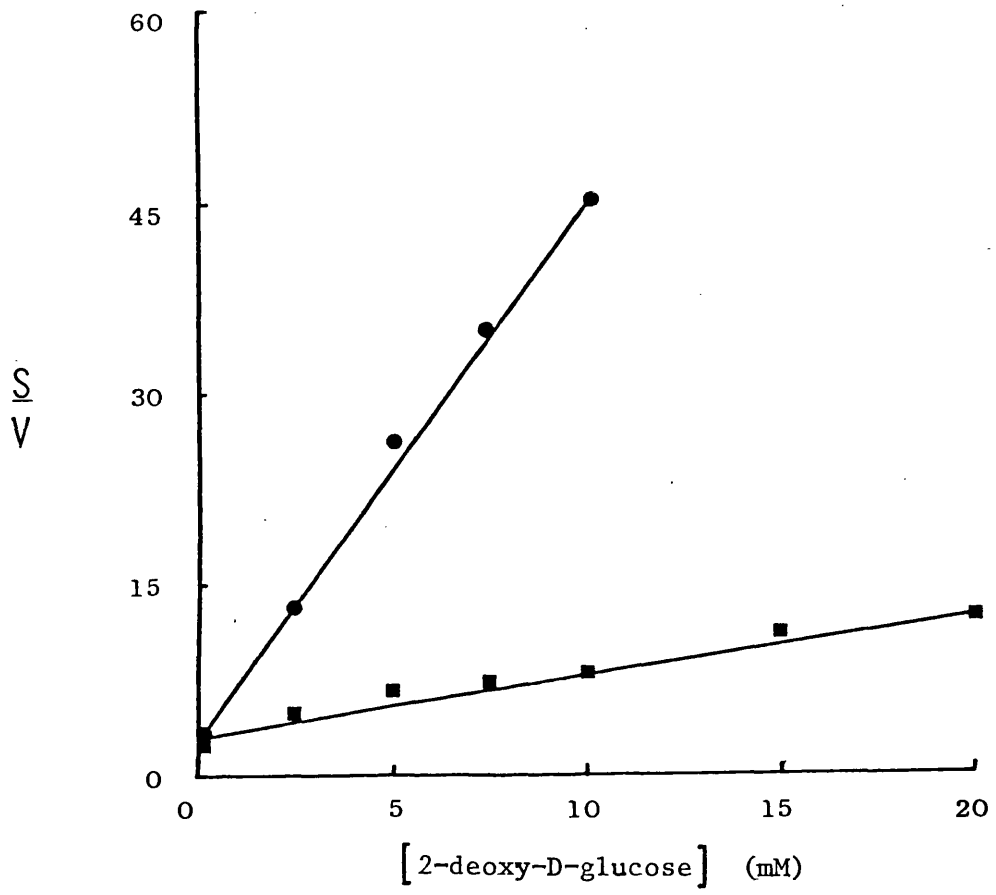
2-deoxy-D-glucose is an analogue of D-glucose which is transported by the D-glucose transport system of the rat adipocyte (Olefsky, 1978). It is a substrate for hexokinase, which converts it to 2-deoxy-D-glucose-6-phosphate, which is only slightly metabolised. Fig. 35 shows the inhibition of D-allose exchange in insulin-stimulated adipocytes in the presence and absence of 2mM potassium cyanide. The cells were incubated

Fig 34.



The inhibition of D-allose transport (in cells treated with 10nM insulin) by 3-O-methyl-D-glucose in the absence of potassium cyanide (●), K_i = 4.75 ± 0.36 mM (n=18); and in the presence of 2 mM potassium cyanide (■), K_i = 4.77 ± 0.48 mM (S.E., n=12).

Fig 35.



The inhibition of D-allose transport (in cells treated with 10nM insulin) by 2-deoxy-D-glucose in the absence of potassium cyanide (●), K_i = 0.74 ± 0.03 mM (S.E., n=15), and in the presence of 2mM potassium cyanide (■) K_i = 5.80 ± 0.44 mM (S.E., n=30).

for 20 minutes with 2-deoxy-D-glucose before estimating the inhibition of D-allose exchange. The Ki for 2-deoxy-D-glucose in control cells is 0.74 ± 0.03mM (n = 15) whilst in cyanide-treated cells it is 5.80 ± 0.44mM (n = 30).

D-glucose-1-phosphate, D-glucose-6-phosphate and 2-deoxy-D-glucose-6-phosphate showed no detectable inhibition of D-allose exchange when added exogenously to adipocytes.

Regulation of hexose metabolism in adipocytes, the relationship to hexose transport and the action of insulin

A number of compounds other than the recognised hormones have been shown to affect hexose metabolism in adipocytes. In order to investigate possible effects of these compounds on hexose transport a number of compounds were screened for effects on hexose transport. In addition a number of these compounds have been implicated in the action of insulin, and these effects have also been studied.

All the test compounds were added exogenously to cells at 0.1mM (final concentration) unless otherwise stated. The compounds were tested for possible effects on basal cells, cells stimulated with 50pM insulin (approximately ½ maximal stimulation) and cells stimulated with 10nM insulin (a ten-fold excess of that required for maximal stimulation (Whitesell & Gliemann, 1979)). In all cases the compound was incubated with the cells for 30 minutes. Transport was assayed by the uptake of 1mM 3-O-methyl-D-glucose in 20 seconds (basal cells), 5 seconds (for half maximal stimulation) and 1 second (fully stimulated with insulin).

These times have been shown by Taylor & Holman (1981) to give a good estimate of the initial rates of uptake at this concentration.

The effects of adenosine and adenosine nucleotides

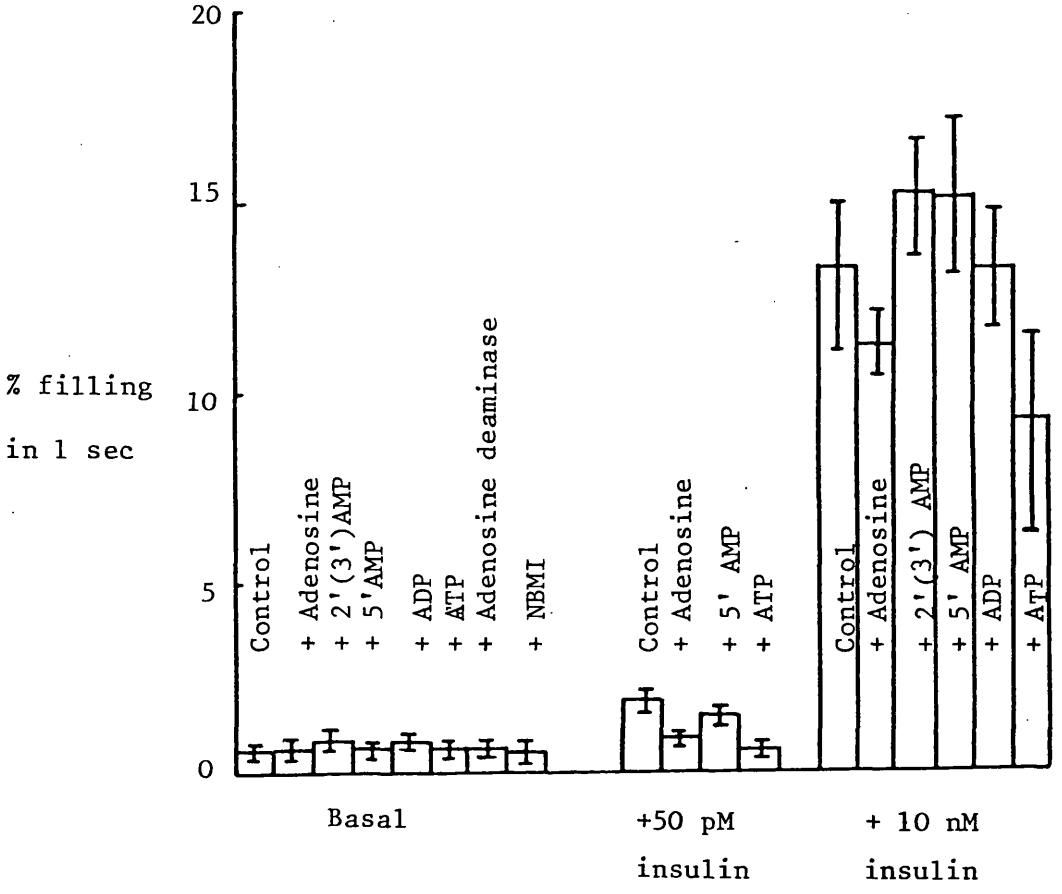
Adenosine and related compounds have been shown to affect the metabolism of adipose tissue (Dole, 1961) and some reports have indicated that the hexose transport system may be regulated by these compounds (Taylor & Halperin, 1976).

Adenosine

Fig. 36 shows the effects of adenosine and its related nucleotides on the rate of 1mM 3-O-methyl-D-glucose uptake. Adenosine deaminase was added at 1IU/ml to the suspending buffer, and the adenosine transport inhibitor NBMI was present at 4μM. Cass & Patterson 1975 showed that 4μM NBMI completely inhibited adenosine uptake in erythrocytes.

The uptake of adenosine by adipocytes was studied using the method described for zero trans entry experiments except that [8-^{3}H] adenosine was used as the radiolabel and 4μM NBMI was used in place of phloretin in the stopping solution. The uptake of 1mM adenosine by adipocytes was found to be rapid with a $t_{\frac{1}{2}}$ = 16.39 ± 1.52 sec (n = 4) in basal cells with a similar $t_{\frac{1}{2}}$ = 16.46 ± 4.27 sec (n = 4) for cells stimulated with 10nM insulin. Adenosine achieved a similar equilibrium volume (1.76μl/100μl packed cells) to that found for 3-O-methyl-D-glucose (1.8μl/100μl packed cells). There was no accumulation of adenosine beyond equilibrium for at least 2 minutes suggesting that the metabolism of adenosine is slower than its transport rate.

Fig 36.



The effect of adenosine analogues on the rate of 1 mM 3-O-methyl-D-glucose uptake in basal adipocytes, adipocytes treated with 50 pM insulin and adipocytes treated with 10 nM insulin. All rates ± S.E. n=4 for all analogues.

No significant effects of 0.1mM adenosine were observed on the rate of hexose transport in either basal or fully-stimulated adipocytes. A significant ($p > 0.005$) reduction of the rate of hexose transport was observed in adipocytes which had been submaximally stimulated with adenosine.

At lower concentrations of adenosine there were small increases in the rate of hexose transport in basal cells (Table 12). The maximal stimulation (14.3%) was observed at 10μM adenosine. Taylor & Halperin (1979) also reported that 10μM adenosine gave a maximal stimulation of glucose oxidation in basal cells but reported a much larger effect (approximately 50% increase).

There was no significant effect of adenosine deaminase on the rate of 3-O-methyl-D-glucose over a prolonged incubation period (60 minutes).

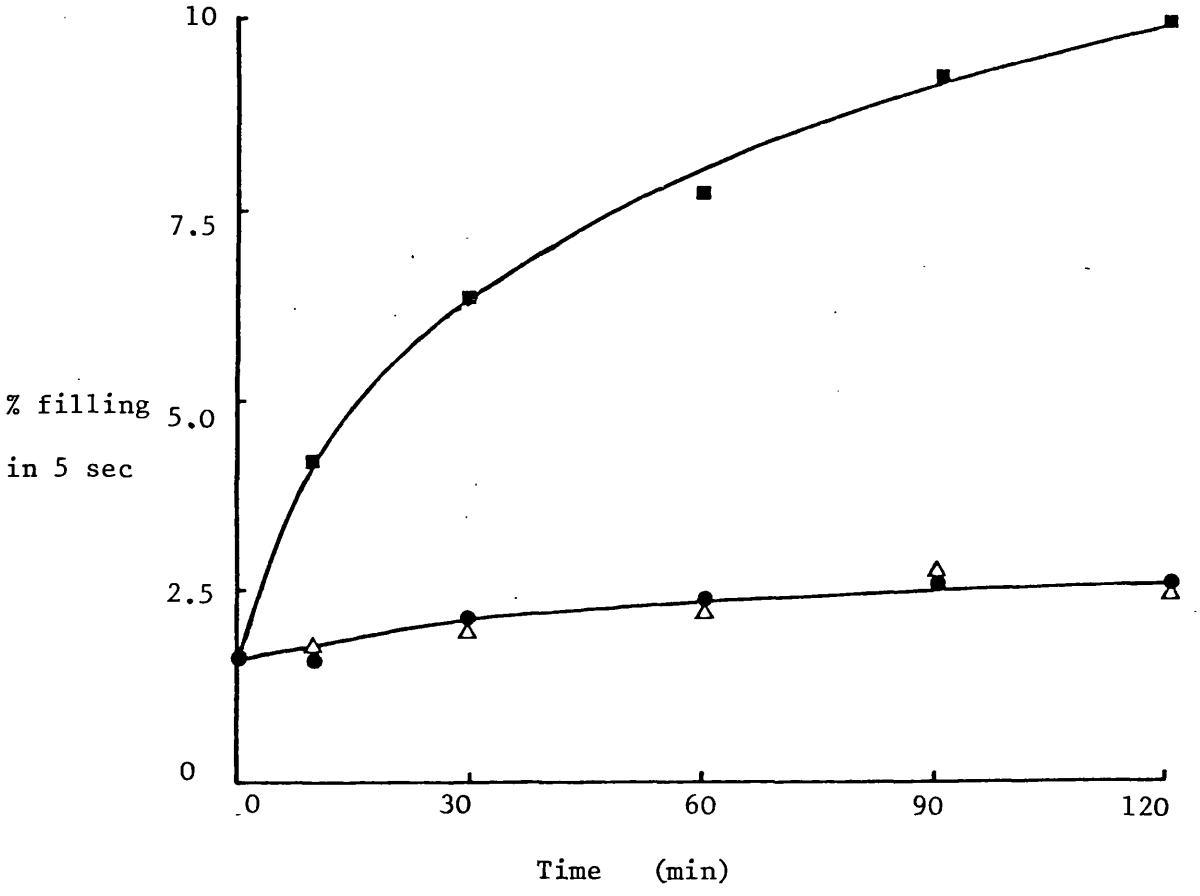
Fig. 37 shows the effects of longer incubations of adipocytes with 0.1mM adenosine and 0.5mM D-glucose. No significant differences between the control and adenosine-treated cells were observed. The slow increase in the rate of 3-O-methyl-D-glucose transport in basal cells is believed to be due in part to the release of fatty acids by the cells.

Also shown in Fig. 37 is the effect of increasing the osmolarity of the suspending buffer by the addition of 400mM mannitol. The results show that a hyperosmolar buffer produces an insulin-like stimulation of hexose transport. Similar results were obtained by Clausen et al. (1970) in both muscle and adipocyte preparations. Whilst the magnitude of the stimulation is similar to that reported by Clausen et al., this experiment shows a slower rate for the activation of hexose transport.

Table 12. The effect of adenosine on hexose transport

Concentration of adenosine	3-O-methyl-D-glucose uptake % filling/second
0	0.658 ± 0.019 (n = 4)
0.1μM	0.488 ± 0.063 (n = 3)
1μM	0.704 ± 0.074 (n = 4)
10μM	0.752 ± 0.069 (n = 4)
0.1mM	0.625 ± 0.029 (n = 4)
1.0mM	0.692 ± 0.091 (n = 4)

Fig 37.



The effect of incubating adipocytes in 1% albumin Hepes buffer containing (●) no addition, (△) 0.1 mM adenosine, (■) 400 mM mannitol, on the rate of 3-O-methyl-D-glucose transport.

The effect of Adenosine 5' phosphates on 3-O-methyl-D-glucose transport

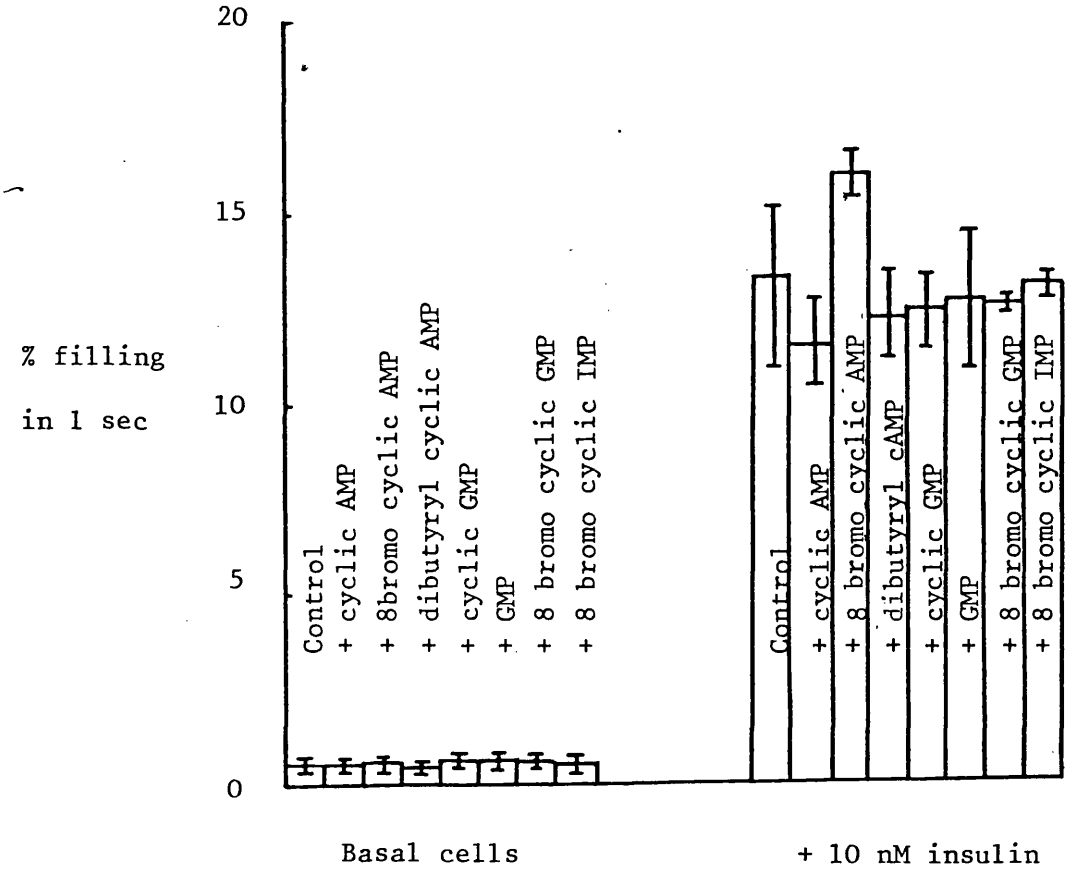
The only adenosine 5' phosphate to show any significant effect was ATP (Fig. 36) which reduced the effects of insulin. In cells stimulated with 50pM insulin the addition of ATP prevented stimulation by insulin ($p > 0.005$). In cells maximally stimulated with insulin the reduction observed is not significant ($p > 0.1$) within the experimental error. There is no significant effect of ATP in basal cells.

The effects of cyclic nucleotides on 3-O-methyl-D-glucose transport

The role of cyclic nucleotides in catecholamine action has been intensively studied, and cyclic nucleotides have been shown to act as the intracellular second messengers for this system. Cyclic nucleotides have also been implicated in the action of insulin. Fig. 39 shows the effects of the addition of a number of cyclic nucleotides and cyclic nucleotide analogues on 3-O-methyl-D-glucose transport in adipocytes. Cyclic nucleotides have been shown to be transported by avian erythrocytes (Davoren & Sutherland, 1963) and human erythrocytes (Holman 1978, 1979) so that a sufficient amount may be able to enter the adipocyte when added exogenously. The lipophilic derivative dibutyryl cyclic AMP (Henion et al., 1972) has also been used to elevate intracellular cyclic AMP levels.

Both cyclic AMP and dibutyryl cyclic AMP show no significant effects in both basal and insulin stimulated adipocytes. There was also no effect of cyclic AMP on cells half maximally stimulated with insulin, control cells showed a rate of 1.97 ± 0.23% filling/second ($n = 4$) whilst with 0.1mM cyclic AMP the rate was 1.67 ± 0.24% filling/second ($n = 4$) which is not significantly different ($p > 01$).

Fig 38.



The effect of cyclic nucleotides and cyclic nucleotide derivatives on the rate of 1 mM 3-O-methyl-D-glucose transport in basal adipocytes and adipocytes treated with 10 nM insulin. All rates ± S.E., n=4 for all analogues.

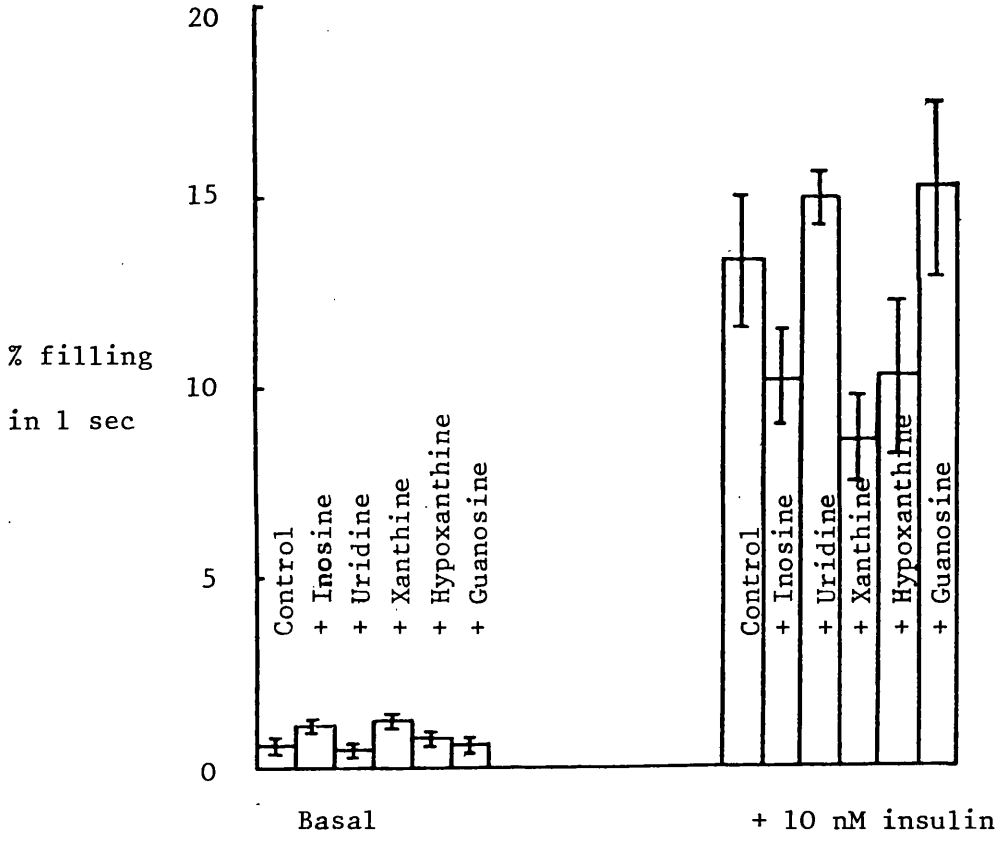
Also shown in Fig. 38 are the effects of guanosine monophosphate cyclic GMP and a range of 8 bromo-substituted cyclic nucleotides. There were no significant effects of any of these compounds.

The effects of nucleosides on 3-O-methyl-D-glucose transport

In addition to adenosine other nucleosides were also tested for effects on 3-O-methyl-D-glucose transport. The results of these experiments are shown in Fig. 39. In basal cells both inosine and xanthine showed significant stimulations of the transport rate ($p > 0.005$ in both cases). None of the other compounds tested showed any significant effects.

In adipocytes stimulated with 10nM insulin, inosine and xanthine show slight inhibition of 3-O-methyl-D-glucose transport. However, within experimental error these results are insignificant ($p > 0.1$ in both cases). Other nucleosides show no effects.

Fig 39.



The effect of purines and purine nucleosides on the rate of 1 mM 3-O-methyl-D-glucose uptake in basal adipocytes and adipocytes treated with 10 nM insulin. All rates ± S.E., n=4 for all analogues.

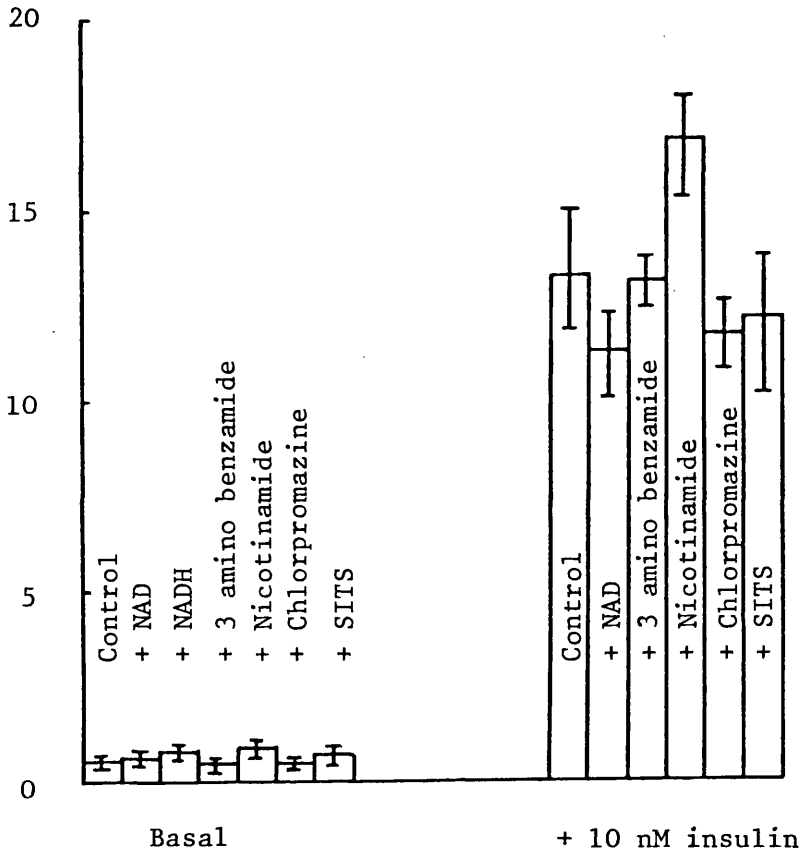
Effects of NAD and ADP ribosylation inhibitors

ADP ribosylation of adipocyte plasma membrane proteins has been shown by Malbon & Gill (1979). In order to investigate whether ADP ribosylation is of importance in the regulation of hexose transport, cells were treated with the ADP ribosylation inhibitors 3-amino-benzamide and nicotinamide. Fig. 40 shows the results of these experiments, with neither of these compounds producing significant changes in the rate of 3-O-methyl-D-glucose transport. There was also no effect on the insulin response when these compounds were added before insulin.

No significant effects of NAD or NADH were observed when these compounds were added to basal cells. There was also no effect of NAD on insulin-stimulated cells (Fig. 12).

Fig. 40 also shows the results of experiments in which the anion transport inhibitor 4-acetamido-4'-isothiocyanato-stilbene-2-2'-disulphonic acid (SITS) was added to adipocytes. There was no significant effect of SITS on basal or insulin-stimulated 3-O-methyl-D-glucose transport in adipocytes. Fig. 40 also shows that chlorpromazine has no effect on basal or insulin-stimulated hexose transport.

Fig 40.



The effects of NAD analogues and ion transport inhibitors on the rate of 1 mM 3-O-methyl-D-glucose uptake in basal adipocytes and adipocytes treated with 10 nM insulin. All rates ± S.E., n=4 for all analogues.

DISCUSSION

Discussion

Hexose transport in the rat epididymal adipocyte is increased by more than 20-fold by insulin. The extreme sensitivity of the adipocyte towards insulin and the relative ease with which pure preparations of adipocytes can be obtained has led to intensive study of this system, in order to investigate the mechanism of hormonal regulation of hexose transport.

The isolated adipocyte preparation

The preparation of isolated adipocytes involves the exposure of cells to a crude mixture of proteases which may alter the characteristics of the cell through proteolysis of the cell surface. It is therefore important to ensure that the isolated cell behaves in a similar manner to the cells in intact tissue. The rate of lipogenesis from D-glucose provides a convenient measure of the rate of metabolism by adipocytes in whole tissue, since cells other than adipocytes would not be expected to show rapid lipogenesis. When whole epididymal fat pads are incubated in a buffer containing radiolabelled D-glucose the addition of a maximal dose of insulin leads to a 10 to 20-fold stimulation of lipogenesis. In the intact tissue many of the cells are not in free contact with the bulk solution and the rate of lipogenesis may be rate limited by the availability of substrates (Gliemann & Vinten, 1974). Insulin is also rapidly degraded by intact adipose tissue (Antoniades & Gershoff, 1966) and therefore some of the adipocytes in whole tissue may be exposed to a submaximal dose of insulin. The results presented in this thesis show that the isolated adipocyte preparation used in these experiments showed a larger increase in the rate of lipogenesis in response to insulin than whole adipose tissue. A similar result was reported by Gliemann (1968) who proposed that the differences were due to the isolated cells being in contact with the bulk solution.

It can therefore be concluded that the metabolism and hormonal responsiveness of the isolated adipocyte is similar to that observed in intact tissue. Since the rate of lipogenesis from low concentrations of D-glucose is limited by the transport of D-glucose into the adipocyte (Gliemann, 1968) the rate of lipogenesis from tracer D-glucose gives a measure of the rate of D-glucose transport. This suggests that the hexose transport rate is also unchanged by isolation.

Many investigators report that some isolated adipocyte preparations show much smaller increases in the rate of lipogenesis or hexose transport in response to maximal doses of insulin. The reasons for this variation are not clear but may be due to a number of factors.

Crude collagenase preparations from different cultures of Clostridium histolyticum show marked differences in the proportions of collagenase and trypsin-like activity present. The presence of small amounts of trypsin-like activity is however required in order to produce complete disruption of the tissue. Purified collagenase preparations are less effective in disrupting tissue. Mild trypsin digestion of the adipocyte leads to marked stimulation of glucose transport (Kono, 1969). This effect is reversible with the cells recovering after 15-20 minutes. Thus the collagenase preparation is most effective when it contains a small amount of trypsin-like activity in addition to collagenase activity. By allowing the cells to wash over a period of 15-20 minutes the effects of trypsin-like activity can also be eliminated. The variability of commercial collagenase preparations (Czech, 1980) requires careful screening in order to select the most effective preparation (see p. 79).

In order that consistent results are obtained adipocyte preparations must be handled carefully. Vega & Kono (1979) showed that mechanical agitation or centrifugation of adipocytes leads to a large increase in the rate of 3-O-methyl-D-glucose transport in the absence of hormones. In most cases the differences in the magnitude of the insulin effect on hexose transport are not due to increases in the rate of insulin stimulated hexose transport but are due to changes in the basal rate. This leads to changes in the magnitude of the insulin response when expressed as a fold stimulation. Thus during the preparation of isolated adipocytes (especially basal cells) it is important to ensure that mechanical agitation is kept to a minimum.

Salans et al. (1981) have shown that dietary changes which lead to an increase in the size of adipocytes (i.e. increased fat content) alter the rate of D-glucose transport. Thus adipocytes prepared from obese animals show increased rates of basal hexose transport, and show a reduced stimulation of hexose transport in response to insulin. The age and diet of the animals used for the preparation of adipocytes will thus influence the magnitude of the stimulation of hexose transport by insulin. In order to eliminate this source of variability animals must be of similar ages and weights and maintained on a uniform diet.

The choice of container for holding adipocyte suspensions is also important. Adipocytes have been shown to adhere to glass surfaces and this is followed by cell lysis (Rodbell, 1961). The adhesion of cells is reduced by the use of siliconising treatments which react with the glass surface to produce an organic silicone layer which gives the surface non-wetting properties. These treatments do not appear to provide the most suitable surface and can only be used for short periods. The use of plastic

vessels to contain adipocyte suspensions offers a more suitable alternative since adipocytes do not adhere to this surface. These materials also appear to absorb free lipid released by cell lysis and hence prevent the rapid lysis of cells that occurs following the release of lipid into the medium. Not all plastics are however suitable for the preparation of adipocytes as some types of plastic appear to release plasticisers and other complex organic molecules into the buffer solution. These compounds have detrimental effects on cells (Cook et al., 1976). Polystyrene and polypropylene were found to be the most suitable plastics for holding adipocyte preparations and these were used wherever possible.

The long-term viability of adipocytes is mainly influenced by the stability of the cells. A high concentration of bovine serum albumin in the buffer was found to be required to prevent a rapid exponential lysis of the adipocytes. Suspensions of adipocytes at a cell density of greater than 40% packed cells were found to be less stable than those at lower cell densities. Since isolated adipocytes have a density less than that of the suspending buffer the cells float to the surface to form a layer of packed cells at the surface. It is important to ensure that adipocyte suspensions are gently resuspended every few minutes in order to prevent this aggregation. The choice of buffer is also important. Marshall & Olefsky (1981) have recently reported that tris(hydroxymethyl)amino methane buffers give an insulin dependent loss of insulin receptors from the cell surface, an effect not observed with other buffering agents.

The cytoplasmic volume of an adipocyte is a very small proportion of the total intracellular volume of the adipocyte and therefore the ratio of surface area to cytoplasmic volume is small when compared with other cell types (Angel & Farkas, 1970). Thus hexoses rapidly equilibrate across the cell membrane and rapid measurements are required in order to measure

initial rates of transport (Whitesell & Gliemann, 1979). Alternatively integrated rate equations which allow for backflux can be used (Taylor & Holman, 1981). Czech (1976a)and Siegel & Olefsky (1980) used relatively long periods to measure the rate of transport and thus these experiments may have underestimated the initial rate of transport (Whitesell & Gliemann, 1979; Taylor & Holman, 1981) with a resulting underestimate in the increase in the rate of hexose transport due to insulin.

Thus the isolated adipocyte preparation is subject to a number of experimental errors which will reduce the apparent effect of insulin. When all these factors are taken into account the insulin stimulation of hexose transport in the adipocyte is probably in excess of 50-fold.

The kinetics of hexose transport in the adipocyte

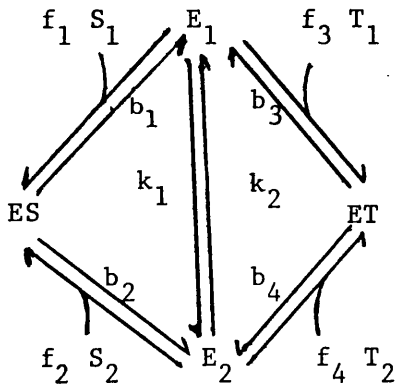
Previous reports by Whitesell & Gliemann (1979), Taylor & Holman (1981) and the results presented in this thesis show that 3-O-methyl-D-glucose is transported symmetrically by the adipocyte hexose transport system. The effect of insulin is to increase the Vmax without affecting the Km. The symmetry of hexose transport by the adipocyte is unaffected by insulin.

The modified method of performing zero trans exit experiments (by measuring the zero time concentration in the presence of 50μM cytochalasin B) gives a more accurate estimation of the internal substrate concentration at zero time. Taylor & Holman (1981) reported a K_{zt}^{io} = 2.66mM for the zero trans exit experiment compared to a K_{ic}^{oi} = 6.51mM for the infinite *cis* experiment which also measures the Km for the internal site. The modified zero trans exit technique gives a K_{zt}^{io} = 5.65 mM which

is very similar to the value for the infinite cis influx experiment of Taylor & Holman. The reason for the discrepancy when phloretin is used to determine the internal substrate concentration at zero time is not clear. This effect may be related to the ability of phloretin to inhibit non-mediated transport through the membrane as shown by the inhibition of 4,6-O-ethylidene-D-glucose uptake by phloretin (see p.145). In influx experiments the error due to this effect of phloretin is minimal with only slight differences between blanks performed using phloretin and cytochalasin B. Only when hexose transport was almost completely inhibited did the difference between phloretin and cytochalasin B blanks become significant (see p.124).

The interaction of two substrates with the hexose transport system

Of the variety of models proposed to account for the transport of hexoses (see p. 37) the simple carrier model is the most widely accepted. When two substrates S and T interact with the carrier this can be represented schematically as



Using a similar symbolism to that described previously (see p.37) in an extension of the analysis of Stein & Lieb (1974a) a unidirectional flux equation can be derived (Rees & Holman, 1981).

$$U_{12}^{S} = \frac{\frac{S_1}{K_S} + \frac{S_1 S_2}{K_S^2} + \frac{S_1 T_2}{K_S K_T}}{R_{oo} + \frac{R_{12}^{S} S_1}{K_S} + \frac{R_{21}^{S} S_2}{K_S} + \frac{R_{12}^{T} T_1}{K_T} + \frac{R_{21}^{T} T_2}{K_T} + \frac{R_{ee}^{S} S_1 S_2}{K_S^2} + \frac{R_{ee}^{T} T_1 T_2}{K_T^2} + \frac{R_{ee}^{ST} S_1 T_2}{K_S K_T} + \frac{R_{ee}^{TS} T_1 S_2}{K_S K_T}} \qquad (12)$$

where:-

$$K_S = \frac{k_1}{f_1} + \frac{k_2}{f_2}$$

$$K_T = \frac{k_1}{f_3} + \frac{k_2}{f_4}$$

$$n\,R_{oo} = \frac{1}{k_1} + \frac{1}{k_2}$$

$$n\,R_{21}^{S} = \frac{1}{b_1} + \frac{1}{k_1} \qquad n\,R_{21}^{T} = \frac{1}{b_3} + \frac{1}{k_1}$$

$$n\,R_{12}^{S} = \frac{1}{b_2} + \frac{1}{k_2} \qquad n\,R_{12}^{T} = \frac{1}{b_4} + \frac{1}{k_2}$$

$$n\,R_{ee}^{S} = \frac{1}{b_1} + \frac{1}{b_2} \qquad n\,R_{ee}^{T} = \frac{1}{b_3} + \frac{1}{b_4}$$

$$n\,R_{ee}^{ST} = \frac{1}{b_1} + \frac{1}{b_4} \qquad n\,R_{ee}^{TS} = \frac{1}{b_2} + \frac{1}{b_3}$$

Thus

$$R_{oo} = R_{21}^{S} + R_{12}^{S} - R_{ee}^{S} = R_{21}^{T} + R_{12}^{T} - R_{ee}^{T}$$

$$R_{ee}^{S} + R_{ee}T = R_{ee}^{ST} + R_{ee}^{TS}$$

$$R_{ee}^{ST} = R_{21}^{S} + R_{12}^{T} - R_{oo}$$

$$R_{ee}^{TS} = R_{12}^{S} + R_{21}^{T} - R_{oo}$$

In the inhibition experiments the substrate (D-allose) was at a low concentration relative to its Km. If $S_o = S_i$ and $T_o = T_i$ in equation (12) then the flux of the radiolabelled substrate (S) is given by

$$U^S_{1-2} = \frac{\frac{S}{R_{oo} K_S}}{1 + \frac{T R^T_{ee}}{K_T R_{oo}}} \qquad (13)$$

From this equation it can be seen that the carrier model predicts that the Ki for T will be equal to the exchange Km for T. Also the inhibition constant for T should be independent of the substrate used. If the assumption of equal inhibitor distribution is not met as is the case for a non-transported inhibitor added externally then the apparent Ki will mainly reflect the affinity of the inhibitor for the external site.

The results presented in this thesis show that the Km's for 3-O-methyl-D-glucose and D-xylose equilibrium exchange in the adipocyte are similar to the Ki's determined by the inhibition of D-allose exchange. This result does not exclude the possibility of multiple site models where substrate and inhibitor can interact with the transporter. The allosteric pore model (Holman, 1980) for example predicts that the ratio of tracer permeabilities (V/K) through the low and high affinity components may be different for different tracers and the Ki's need not equal the equilibrium exchange Km's. If however the V/K ratios for the low and high affinity forms are approximately equal then only a single Km (or Ki) will be apparent (Holman et al., 1981).

Holman et al. (1981) reported evidence for negative cooperativity in hexose transport in the human erythrocyte. The results of Holman et al's experiments revealed non-linearity in plots of S/V vs S for D-glucose exchange in human erythrocytes. Similar non-linearities were also observed

in the inhibition of tracer 3-O-methyl-D-glucose, D-fructose and D-xylose exchange in human erythrocytes. The results indicated the presence of two apparent affinity constants of 2mM and 26mM for D-glucose. There was less evidence for non-linearity in equilibrium exchange and inhibition experiments in the adipocyte with the equilibrium exchange of 3-O-methyl-D-glucose and the inhibition of D-allose exchange by 3-O-methyl-D-glucose showed linear reciprocal plots over a wide range of 3-O-methyl-D-glucose concentrations. The significance of this result will be discussed further on p. 208.

Hydrogen bonding requirements for binding to the insulin stimulated hexose transporter of adipocytes

From the Ki values for inhibition of D-allose transport of the analogues tested it is possible to construct a model for sugar binding to the transporter (Fig.41).

The high affinity of β-fluoro-D-glucose and the transport of 1-deoxy-D-glucose, which are both fused pyranose ring analogues of D-glucose, indicate that the transporter accepts the substrate in pyranose ring forms rather than in the open chain form. Studies on hexose molecules in solution using nuclear magnetic resonance techniques have indicated that the α and particularly the β forms of all common monosaccharides exist predominantly in the $^4C_1$ conformation (Ferrier & Collins, 1972). Le Fevre (1961) has proposed that the selectivity of the transport system depends on the conformation of the hexose molecule, however since all the analogues used in the experiments described in this thesis will exist predominantly in the $^4C_1$ conformation selectivity based on the stability of different conformations is unlikely. The relative affinities thus reflect hydrogen bonding

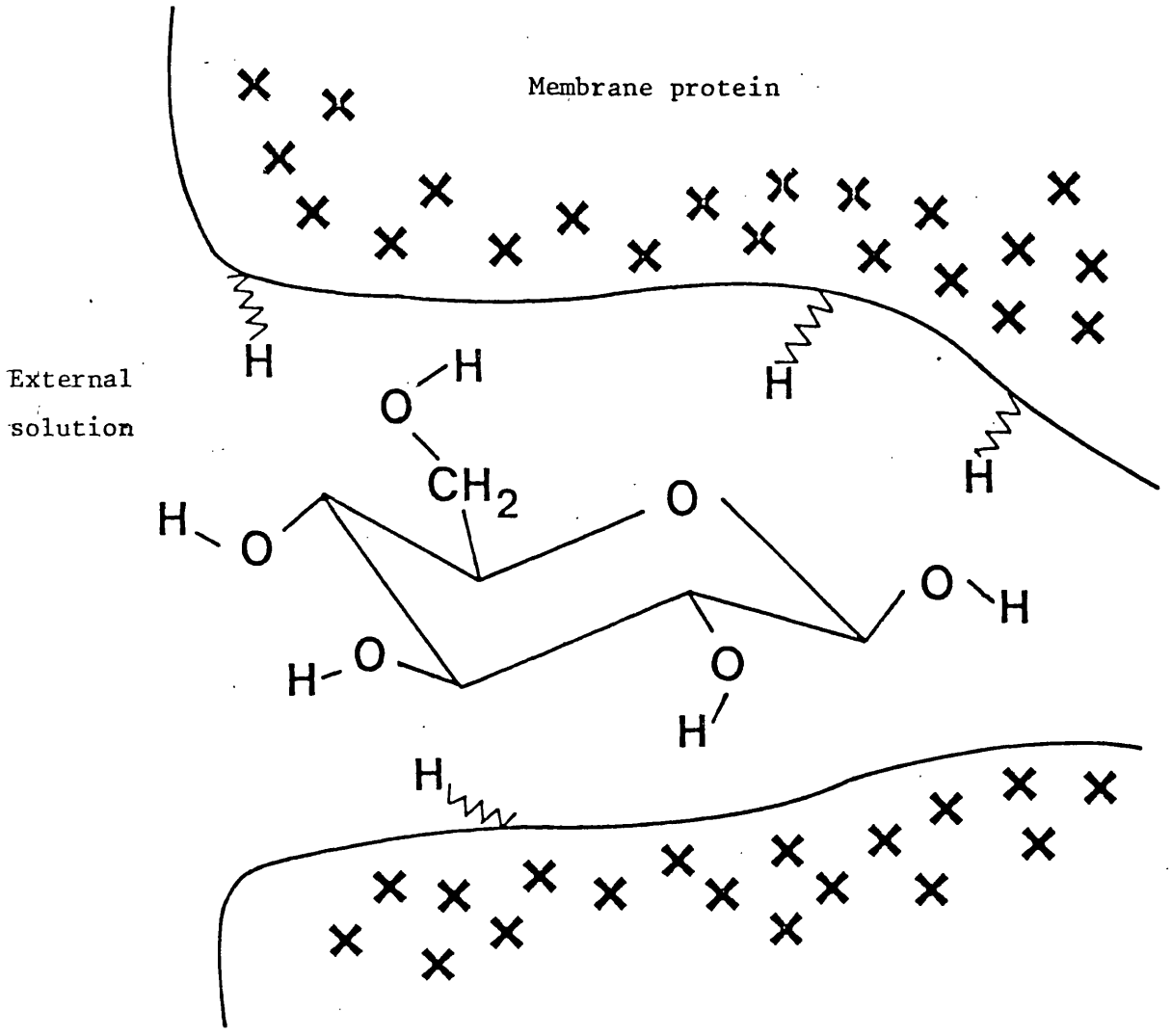


Fig. 41.

The scheme shows the proposed features of the active site at the external surface of the sugar transporter in adipocytes. The important hydrogen bonding sites are the ring oxygen, C-1, C-3 and to a lesser extent C-6. A close approach of the transporter to the sugar at C-1 and C-2, and to a lesser extent at C-3 and C-6 but not at C-4 is suggested. The perferred conformation is $^{4}C_{1}$ glucopyranose.

to specific groups on the transporter rather than the relative stabilities of hexose forms.

The important hydrogen bonding positions on the sugar molecule appear to be the ring oxygen, the C-1 hydroxyl, a gluco- configuration hydroxyl at C-3, and to a lesser extent a hydroxyl at C-6. The C-2 hydroxyl is unimportant and in the manno-configuration may cause steric hindrance. The role of the C-4 hydroxyl in binding is not as clear as for the other positions but may become important in the absence of a hydroxyl at C-6.

In many respects therefore the hydrogen bonding requirements of the adipocyte hexose transport system closely resemble those of the human erythrocyte (Barnett et al., 1973a; Kahlenberg & Dolansky, 1972), the blood brain barrier (Betz et al., 1975) and the sodium independent hexose transporter in the basal-lateral membranes of intestinal cells (Wright et al., 1980). The adipocyte system differs from the erythrocyte system principally in the apparent lack of specificity at the C-4 and C-6 positions. There are also smaller differences between the affinities for D-glucose and D-galactose (and between analogues in the D-glucose series compared to the D-galactose series) in the rat adipocyte and the human erythrocyte. The loss of the C-6 hydrogen bond is also less important in the adipocyte when compared to the human erythrocyte system.

Interpretation of the results in terms of ascribing specific hydrogen bonding groups is not straightforward, since binding may not be to a single site. Thus although hydrogen bonds are at some stage involved at the ring oxygen, C-1, C-3 and C-6 hydroxyls, these positions need not necessarily form hydrogen bonds to the transport system simultaneously,

and sharing of hydrogen bonding groups as the sugar is translocated may occur. Also in solution hexoses will exist with the ring oxygen and hydroxyl groups hydrogen bonded to water molecules, and changes in the hydration shell of the molecule may affect the apparent affinity constants.

Spatial requirements for hexose binding to the insulin sensitive adipocyte hexose transporter

The use of hexoses alkylated at the various hydroxyls has been used as a measure of the space available around the different hydroxyl groups when the hexose is bound to the transport system. In order to investigate the features of the outside site inhibitors were added with substrate to the external solution.

In these experiments the important spatial restrictions to binding were at C-1 and C-2, but not at C-4 and C-6. This implies a strict orientation of the sugar molecule in the outside active site with C-1 of the sugar facing inward. This result is very similar to that reported for the human erythrocyte by Barnett et al. (1973b, 1975), with very small differences, the adipocyte having a somewhat 'looser' fit at C-1 and C-3 as compared to the erythrocyte since in the adipocyte α-methyl-D-glucoside and 3-O-propyl-D-glucose show more affinity as inhibitors than in the erythrocyte. This difference is however small when compared with the very clear difference in spatial requirements between C-1 and C-4 shown by both systems.

In order to study the inside requirements for binding to the transport system, it is necessary to prepare analogues which can enter the cell independently of the transport system. Alkyl substituted D-glucose analogues

inhibit both the erythrocyte and adipocyte transport system but are not transported by it. The rate of uptake of alkyl sugars into adipocytes is dependent upon the size of the lipophilic substitution, thus methyl-β-D-glucose is taken up 27 times slower than n'-propyl-β-D-glucoside. The rate of methyl-β-D-glucoside uptake probably represents the lowest limit for non-mediated uptake (Vinten, 1978). The uptake of alkyl sugars is through a route which is insulin-insensitive and not inhibited by 50mM D-glucose. The results show the uptake of 4,6-O-ethylidene-D-glucose to be very temperature-sensitive and to be slightly inhibited by phloretin. The effect of phloretin on membrane fluidity (Plagemann, 1981) may explain the apparent inhibition of 4,6-O-ethylidene-D-glucose entry by phloretin. These results suggest that alkyl sugars enter the adipocyte via a non-mediated route.

The asymmetric inhibition of the β-alkyl sugars and 4,6-O-ethylidene-D-glucose indicate that the inner and outer binding sites for hexoses on the transport system are arranged so that the orientation of the sugar molecule is conserved as it passes from one site to the other. Thus at both inner and outer sites C-1 faces the internal solution whilst C-4 faces the external solution. On the basis of this information it is possible to construct a model for the interactions of the hexose molecule with the transporter (Fig. 42).

The poor inhibition of 6-O-propyl-D-galactose is in contrast to the human erythrocyte, since in the erythrocyte system this analogue has a high affinity for the outside site (Barnett et al., 1973b, 1975). This, in conjunction with the weaker hydrogen bond to C-6, indicates that the adipocyte transport system has a slightly different structure to the erythrocyte system at the C-6 binding position.

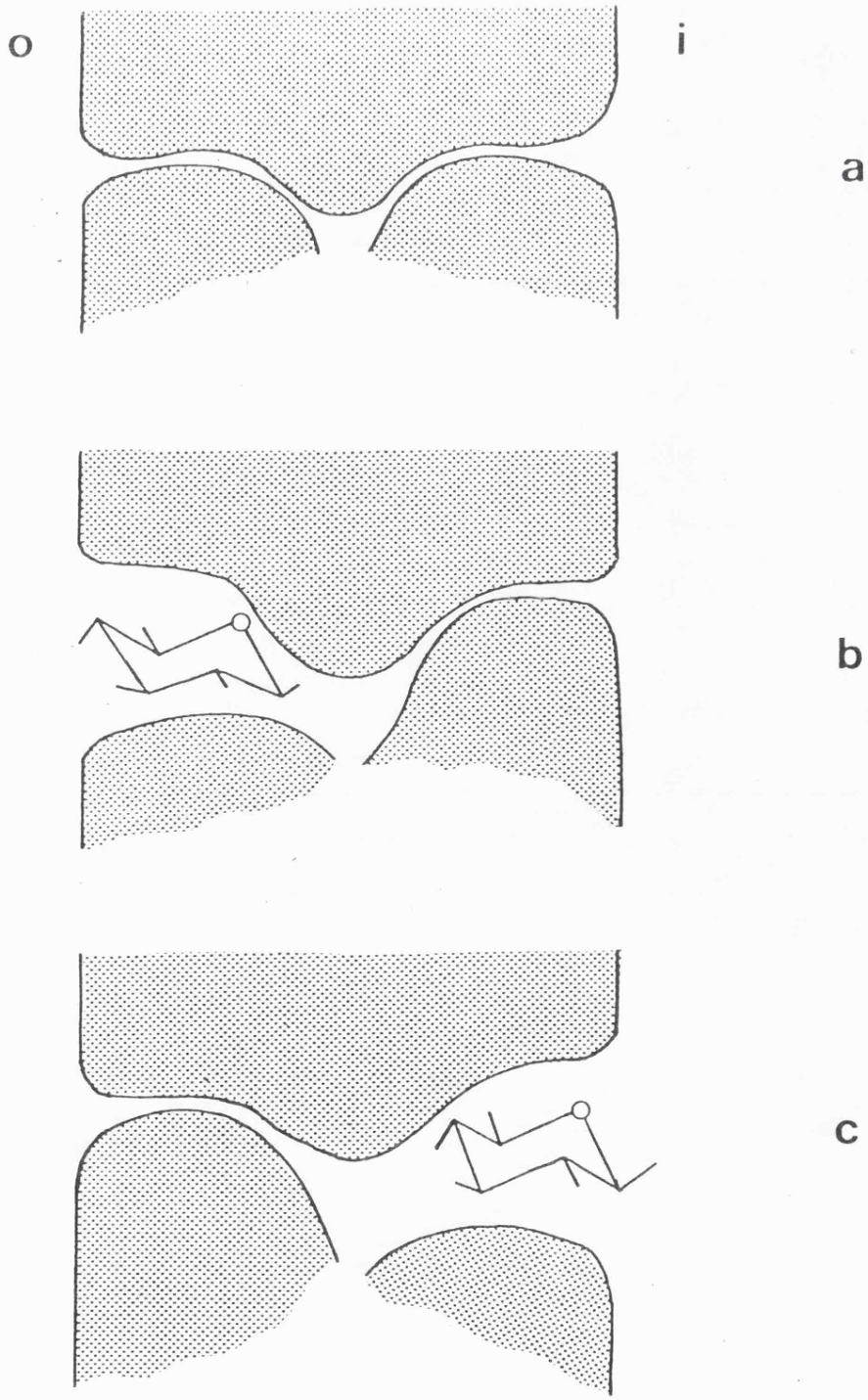


Fig. 42.

The proposed structure of the transporter. (a) In the absence of substrate the system is closed. (b) Binding to the external site destabilises the interface between subunits. Sufficient space is available to accommodate a bulky group at C-4. (c) Binding to the internal site opens the internal subunit interface. Sufficient space is available to accommodate a bulky group at C-1.

The presence of hydrophobic regions immediately adjacent to both the inside and outside hexose binding sites of the adipocyte is suggested by the increased affinity of hexose analogues with hydrophobic substitutions at C-1 and C-4/C-6. Thus the Ki of C-1 alkylated D-glucose analogues decreases in the order n'-propyl > n'-butyl > phenyl. Similarly 6-O-pentyl-D-galactose is a much better inhibitor than 6-O-propyl-D-galactose. A similar hydrophobic region was proposed to be present adjacent to the external site of the human erythrocyte hexose transport system by Barnett et al.,(1973b, 1975). Novak & Le Fevre (1974) also reported that sugars with large hydrophobic substitutions were good inhibitors of hexose transport in the erythrocyte although their results did not show the clear spatial differences reported by Barnett et al. (1973b, 1975). The results of Novak & Le Fevre may be due to hydrophobic binding through the alkyl groups rather than hydrogen bonding to the glucose moiety. A major problem with the use of highly substituted D-glucose analogues is the detergent-like properties of these molecules which reduces the potential uses of these compounds.

Other sugar analogues which have been tested for side specific properties in the human erythrocyte show poorer side specific properties and have a high Ki for transport indicating weak binding to the transporter. 1,2-isopropylidene-D-glucofuranose is not side specific (Baker et al., 1978, Krupka & Devés, 1980). This is probably because this compound exists as a furanose ring whereas the site requires a pyranose ring (Kahlenberg & Dolansky, 1972; Barnett et al., 1973a). Methyl-2,3-di-O-methyl-α-D-glucoside (Baker et al., 1978; Widdas, 1980) is also a poor side specific analogue with a high Ki for the inhibition of hexose transport in the human erythrocyte.

The presence of hydrophobic regions adjacent to the site may also account for the very high affinity of the competitive inhibitors phloretin (Jennings & Solomon, 1976) and cytochalasin B (Basketter & Widdas, 1978; Vinten, 1978). Taylor & Gagneja (1975) put forward stereochemical evidence for cytochalasin B having a spatial distribution of four oxygen atoms similar to those found in the $^4C_1$ conformation of D-glucose and proposed that these atoms may participate in the binding of cytochalasin B to the active site of the hexose transporter. Hydrogen bonding to these positions does not account for the very high affinity of cytochalasin B and additional hydrophobic interactions around the active site may therefore be involved. The very low Ki of cytochalasin B in the adipocyte, reported to be 0.25μM by Vinten (1978) when compared to D-glucose Ki 8.62mM, suggests that a major proportion of the binding to the hexose transporter may be via hydrophobic interactions. If this is the case the value of cytochalasin B as a side specific analogue directed towards the internal active site is reduced.

Metabolite regulation of hexose transport

Regulation of metabolism via feedback inhibition of an early step in a metabolic pathway is a well-recognised phenomenon (Newsholme & Start, 1973). Since the transport of D-glucose into the cell represents the initial step in its metabolism regulation of D-glucose transport offers the potential for efficient control of metabolism. In adipocytes which are maximally stimulated with insulin the transport of D-glucose ceases to be the rate-limiting step in lipogenesis when the external D-glucose concentration exceeds 1 to 2mM (Gliemann, 1968, Czech, 1976). Hales & Randle (1963) reported that the blood glucose level in fed rats was 147mg/100ml (8.2mM) and that the serum insulin level was 130μU/ml (approximately 800pM). Whitesell & Gliemann (1979) reported that 3-O-methyl-D-glucose transport in the isolated rat adipocyte was maximally stimulated by 200pM insulin. Thus in the post-absorbtive state hexose transport may not be the rate-limiting step for lipogenesis. Despite the large stimulation of the rate of hexose transport in the adipocyte it is therefore possible that insulin may offer only a coarse regulation of hexose metabolism through changes in the transport rate. If this is the case metabolite regulation of hexose transport may offer an important fine control of hexose metabolism.

The results presented in this thesis indicate that phosphorylated sugars may interact with the transporter in a different manner to non-phosphorylated sugars. There is a marked reduction in the inhibition of D-allose exchange when the phosphorylation of D-glucose and 2-deoxy-D-glucose is inhibited by cyanide poisoning, whereas the inhibition of D-allose exchange by 3-O-methyl-D-glucose is unaffected.

Kono et al. (1977) reported that ATP depletion does not affect the transport of the non-phosphorylated D-glucose analogue 3-O-methyl-D-glucose in the adipocyte. Thus the effects of intracellular ATP depletion are linked to changes in the intracellular concentration of metabolites of the transported hexose and not to a generalised requirement of ATP for transport. This result also eliminates the possibility of a direct effect of cyanide on the hexose transport system. The largest effect of ATP depletion is on the inhibition of D-allose exchange by 2-deoxy-D-glucose. In normal adipocytes this analogue is principally converted to 2-deoxy-D-glucose-6-phosphate with very small amounts of 6-phospho-2-deoxy-gluconate also being formed (Olefsky, 1978). This result suggests that the effects observed are due to sugar-6-phosphates or 6-phosphogluconates, although effects due to other 2-deoxy-D-glucose metabolites which may be effective at very low concentrations cannot be ruled out.

The lack of inhibition of D-allose exchange by sugar phosphates added externally is not surprising, since these charged molecules would not be expected to penetrate the plasma membrane.

Foley et al. (1980a) have also reported anomalies in the transport of phosphorylated sugars in adipocytes. Foley et al. reported a three-fold acceleration of tracer (a concentration very much lower than the Km) 2-deoxy-D-glucose uptake following a 30 minute pre-exposure of insulin stimulated adipocytes to 10mM D-glucose or D-mannose. There was no accelerating effect with non-phosphorylated sugars or non-hexose metabolites. The accelerating effect was reduced in basal adipocytes (1.5 fold increase) consistent with the reduced intracellular concentrations of glucose and its metabolites in the absence of insulin (Foley et al., 1980).

The acceleration of tracer 2-deoxy-D-glucose uptake required a 5 minute pre-exposure to D-glucose and the threshold glucose concentration which gave the effect was 2.5mM. These results were interpreted by Foley et al. as an acceleration of the transport of 2-deoxy-D-glucose through a change in the Vmax, and they suggested that these results were not due to changes in the rate of 2-deoxy-D-glucose phosphorylation since their methods measured the total 2-deoxy-D-glucose uptake in 2 seconds.

Foley & Gliemann (1981) have also reported that adipocytes can accumulate free intracellular 2-deoxy-D-glucose against its concentration gradient. They also reported that a high extracellular concentration of 2-deoxy-D-glucose accelerates the efflux of 2-deoxy-D-glucose from adipocytes suggesting a rapid dephosphorylation reaction. From their results Foley & Gliemann (1981) proposed that the dephosphorylation of 2-deoxy-D-glucose-6-phosphate occurs in a cellular compartment where the newly-formed 2-deoxy-D-glucose cannot rapidly diffuse to the transport system.

From their results Foley et al. (1980a) proposed a model for hexose transport in the adipocyte in which two in-series rate-limiting barriers control the uptake of hexoses. Foley et al. proposed that one barrier is rate-limiting for 3-O-methyl-D-glucose transport whilst the other is partially rate-limiting for 2-deoxy-D-glucose in the absence of metabolites.

The results presented in this thesis show increased inhibition of D-allose exchange by phosphorylated sugars. These results do not reflect the rates of transport of these sugars and also do not differentiate between an increased affinity of the transported sugar for the transport system in the presence of metabolites or the inhibiton of

hexose transport by metabolites. Foley et al. (1980a) report only a slight accelerating effect when adipocytes are preincubated with 2-deoxy-D-glucose and on the basis of this result suggest that the metabolite responsible for regulation is not a sugar-6-phosphate. In contrast the results presented in this thesis indicate that of the phosphorylated sugars 2-deoxy-D-glucose is the most sensitive to the depletion of ATP.

Inhibition of hexose transport by sugar-6-phosphates or an allosteric interaction of sugar-6-phosphates with the hexose transport system may provide a possible explanation of the results of Foley et al. D-glucose will compete with 2-deoxy-D-glucose for hexokinase leading to a reduction in the size of the sugar-6-phosphate pool (since glucose-6-phosphate will be rapidly metabolised) which leads to an increased rate of hexose transport. 2-deoxy-D-glucose will not affect the size of the sugar-6-phosphate pool and will therefore have no effect. Direct competition of sugar-6-phosphates for the inside site of the hexose transporter is unlikely due to the spatial requirements of this site which make it unable to accept sugar molecules with a bulky group at C-6. Any regulation of hexose transport (either acceleration or inhibition) by a hexose metabolite would therefore be expected to occur through either a separate allosteric modifier site or through chemical modification of the transporter using a sugar metabolite as the substrate.

Models for hexose transport

A number of mammalian sodium independent hexose transport systems have now been studied with respect to their substrate specificities. The results of experiments on the human erythrocyte (Barnett et al., 1973a. Kahlenberg & Dolansky, 1972), basal lateral membranes from the small intestine (Wright et al., 1980), the brain (Betz et al., 1975) and the rat adipocyte (the results presented in this thesis) show that the substrate specificity (and hence the hydrogen bonding requirements) are very similar in all these tissues. In other tissues where detailed analogue studies have not been performed the commonly used sugar analogues show an order of affinities similar to those observed in the characterised tissues, suggesting similar hydrogen bonding requirements. The spatial requirements for hexose transport in the human erythrocyte (Barnett et al., 1973b, 1975) and the rat adipocyte (the results presented in this thesis) are also very similar. It therefore seems reasonable that all the mammalian sodium independent hexose transport systems share a common mechanism of action.

A similar situation is also found with the mammalian sodium dependent hexose transport systems of the intestinal and kidney brush border membranes. Both these systems are similar to one another but differ markedly from the sodium independent hexose transport systems (Silverman, 1976).

Despite the similarity of substrate specificity, mammalian sodium independent hexose transport systems from different tissues differ with respect to the hormonal regulation of transport and the symmetry of the kinetic parameters describing transport. Thus hexose transport in the adipocyte is extremely sensitive to hormonal stimulation whereas in the erythrocyte there is little evidence for hormonal control of transport.

Current views on the mechanism of hormonal stimulaton of hexose transport favour the proposal of Wardzala et al. (1978) that hormonal stimulation leads to an increased number of functional transporters within the membrane rather than a change in the transporter itself. Thus hormonal regulation may be independent of the mechanism of hexose transport. The adipocyte and human erythrocyte hexose transport systems also differ in the symmetry of transport. In the human erythrocyte zero trans entry has a low Km and Vmax, zero trans exit has a high Km and Vmax, and equilibrium exchange also shows a high Km and Vmax. In contrast the rat adipocyte shows similar Km and Vmax values regardless of the experimental protocol used. Thus hexose transport in the human erythrocyte is asymmetric whilst the rat adipocyte is symmetrical.

It has been suggested that the asymmetry of hexose transport in the human erythrocyte arises because uptake is limited by bulk association with haemoglobin within the cell (Holman & Naftalin, 1975, Baker & Naftalin, 1979). Thus a symmetrical transport system will show apparent asymmetry. Challis et al. (1980) reported that the removal of 95% of the haemoglobin from human erythrocytes was without effect on the asymmetry of transport, suggesting that the association of hexoses with haemoglobin does not affect the transport parameters. Rabbit erythrocytes (Regen & Morgan, 1964) show symmetrical transport parameters further supporting the proposal that the asymmetry of the human erythrocyte hexose transport system is a property of the transport system rather than an effect produced by the cell contents.

It is therefore reasonable to propose that a model for the transport of hexoses across mammalian cells should be able to encompass both symmetrical and asymmetrical transport systems without a major change in the mechanism. The kinetic models which have been proposed

to account for the experimental observations can be divided into sequential and simultaneous models. According to sequential models a single substrate molecule can bind to a single substrate binding site which is sequentially available on either face of the membrane. In contrast simultaneous models allow substrate molecules to bind simultaneously to substrate binding sites on both faces of the membrane, to form a ternary complex.

Recently Krupka & Devés (1981) have proposed that D-glucose transport in the human erythrocyte occurs by a sequential process. In their experiments Krupka & Devés studied the inhibition of D-glucose exit by mixtures of phloretin and cytochalasin B. They interpreted their results as being consistent with a sequential model in which phloretin and cytochalasin B are unable to bind to the transporter simultaneously. These experiments were however performed using a very high concentration of D-glucose (124mM) inside the cells which is well above the half saturation constant for the inside site. The inside site will therefore be saturated with D-glucose greatly reducing the inhibition by cytochalasin B (Basketter & Widdas, 1978). Basketter & Widdas (1978) reported that phloretin is not a good side specific analogue and suggested that phloretin may be able to bind to both sides of the membrane.

Sogin & Hinkle (1980) studied the binding of cytochalasin B to the human erythrocyte hexose transporter which had been reconstituted into liposomes and reported that cytochalasin B binding was reduced in the presence of D-glucose. They also reported that the side specific transport inhibitors used by Barnett et al. (1973b; 1975), n'-propyl-β-D-glucoside (inside site) and 6-O-propyl-D-glucose (outside site) also reduced the binding of cytochalasin B. This result suggests that the inner and outer sites are able to interact.

Gorga & Lienhard (1981) have studied the kinetics of cytochalasin B binding to fragments of the human erythrocyte membrane. The membrane fragments were treated with sodium hydroxide and EDTA in order to remove a second high affinity cytochalasin B binding site which is not sensitive to D-glucose (Jung & Rampal, 1977). Gorga & Lienhard reported that cytochalasin B binding was competitively inhibited by D-glucose, n'-propyl-β-D-glucoside and 4,6-O-ethylidene-D-glucose. They were unable to find any evidence for a stable ternary complex being formed between cytochalasin B and 4,6-O-ethylidene-D-glucose, n'-propyl-β-D-glucoside or D-glucose. Gorga & Lienhard concluded that although this result does not exclude models in which there is simultaneous occupancy of the transporter by two or more substrate molecules this observation does restrict the ternary complex to one of very low stability.

Studies on the D-glucose transport system of the human erythrocyte which was reconstituted into liposomes (Sogin & Hinkle, 1978, and Baldwin et al., 1979) have suggested that the stoichiometry of cytochalasin B binding is approximately 0.5 cytochalasin B binding sites per polypeptide chain. More recent studies (Baldwin & Lienhard, 1981) using a different detergent preparation suggest that the stoichiometry of binding may be higher at 0.8 binding sites per polypeptide chain and these authors have suggested that the ratio may be unity.

The transport activity of reconstituted preparations is however low with only 5% of the activity of the intact erythrocyte being expressed. Baldwin & Lienhard (1981) have suggested that this is due to a reduced turnover number and not denaturation of the transport protein(s) during purification. The reason for the reduced turnover number is not clear but may be due to a poor reconstitution into liposomes (Baldwin & Lienhard, 1981).

Jung et al. (1980) have studied the structure of the hexose transporter in the human erythrocyte plasma membrane by radiation inactivation analysis and have suggested that the functional transporter has a molecular weight of 200,000 daltons. In contrast gel electrophoresis shows the cytochalasin B binding protein isolated from human erythrocyte membranes to have a molecular weight of 46,000 daltons (Gorga et al., 1979). Thus the results reported so far suggest that the transporter may exist as a complex of subunits within the membrane.

The most widely used kinetic model for hexose transport is that of the carrier (Widdas, 1952) in which a hexose molecule can bind to a site which is sequentially available on either side of the membrane. In its simplest form the carrier model proposes equal half saturation constants for the binding of substrate on both internal and external faces of the membrane. If the rates of movement of the carrier across the membrane are equal when the carrier is loaded or unloaded then this model fits the observed symmetrical transport parameters of the rat adipocyte (Taylor & Holman, 1981).

In order for the simple carrier model to show accelerated exchange transport as observed in the human erythrocyte it is necessary to postulate different rates of translocation for loaded and empty carriers. Lieb & Stein (1972) have shown that this model predicts that the Km for the infinite cis experiment should be greater than or equal to one half of the Km for equilibrium exchange. In the human erythrocyte the Km for the infinite cis procedure is at least an order of magnitude smaller than the Km for equilibrium exchange. This model also cannot account for the asymmetric Km and Vmax values observed for zero trans entry and exit in the human erythrocyte. Gorga & Lienhard (1981) have proposed that the

asymmetry of hexose transport in the human erythrocyte may be due to the fact that D-glucose is a mixture of α and β-D-glucose forms. Whilst this may in part explain the observations made in the human erythrocyte it is incompatible with the symmetrical transport parameters observed in the adipocyte which would have to show asymmetry of hexose transport in order to cancel out this effect.

Geck (1971) proposed an asymmetric carrier model with different half saturation constants for the inner and outer substrate binding sites in addition to differential mobilities of empty and loaded carriers. In the human erythrocyte the Km's for infinite cis entry and infinite trans exit experiments are low (both experiments measure the Km for the inside site)(Hankin et al., 1972 and Baker & Naftalin, 1979) and therefore inconsistent with the predictions of this model which predicts high Km values for these experiments (Lieb & Stein, 1972). The additional assumption of unstirred layer effects (Regen & Tarpley, 1974) requires large unstirred layers in order to produce significant effects (Lieb & Stein, 1972). Under conditions where the active site is close to the surface of the membrane the unstirred layers would have to be impossibly large. Unstirred layers may however be important if the active site is deeply embedded in the membrane or in a water-filled pore model such as that proposed by Foley et al. (1980a)(see p. 48).

The inhibition constants for the inhibition of hexose transport in the rat adipocyte by the side specific analogues described in this thesis are very similar to the inhibition constants reported for these analogues in the human erythrocyte. Baker et al. (1978) reported the Ki's for 4,6-O-ethylidene-D-glucose inhibition of 2mM 3-O-methyl-D-glucose exchange in the human erythrocyte at 16°C to be 14mM for the outside site and approximately 120mM for the inside site. In the adipocyte the Ki for

4,6-O-ethylidene-D-glucose inhibition of 40μM 3-O-methyl-D-glucose exchange at 37°C is 6.4mM when the inhibitor is outside only and is unchanged when the inhibitor has equilibrated across the cell membrane. Barnett et al. (1975) reported a Ki of approximately 20mM for n'-propyl-β-D-glucoside inhibition of D-glucose exit in human erythrocytes at 18°C. In the adipocyte the Ki for n'-propyl-β-D-glucoside inhibition of 40μM 3-O-methyl-D-glucose exit is 8.88mM at 37°C. These results suggest that the affinities of these inhibitors for the inside and outside sites are similar in both symmetrical and asymmetrical systems. There is no evidence for the ten-fold difference in the affinities of the inside and outside sites in the erythrocyte which would be expected on the basis of a model which predicts asymmetric affinities for the human erythrocyte and symmetrical affinities for the adipocyte.

Carrier models also lack a possible locus for the action of a glucose metabolite (Foley et al., 1980a). The difference in the apparent Vmax values for 2-deoxy-D-glucose and 3-O-methyl-D-glucose transport in the adipocyte reported by Foley et al. is also incompataible with a simple carrier model.

Whilst carrier (sequential) models are consistent with the data of Gorga & Lienhard (1981) and account for much of the kinetic data for hexose transport in the adipocyte and the erythrocyte, anomalies between the predictions of these models and the experimental observations remain. These anomalies are sufficient to reject these models as they are presently postulated, and in view of this a number of simultaneous models have been proposed. These models allow for multiple occupancy of the transporter by substrate.

Pore models predict stable ternary complexes which are incompatible with the observations of Gorga & Lienhard (1981) since the two hexose binding sites are independent of one another. The polar creep model of Bowyer & Widdas (1956) has kinetic predictions which are similar to the asymmetric carrier and this model is subject to the same inconsistencies described previously. The pore model of Naftalin (1970) predicts symmetrical transport parameters and therefore cannot account for the observation of asymmetry in the human erythrocyte.

Ginsburg & Stein (1975) reported the presence of two operational affinities for D-glucose on the inside face of the human erythrocyte membrane. They also suggested that the external site of the human erythrocyte membrane may also show two operational constants. The tetramer model of Lieb & Stein (1970) predicts the presence of two affinity constants on both faces of the membrane. There is less evidence for two affinities in the adipocyte since the infinite cis and zero trans Km's are similar (Taylor & Holman, 1981). The tetramer model is unable to account for the asymmetry of Vmax values for zero trans entry and zero trans exit experiments in the human erythrocyte. The tetramer model also predicts that a stable ternary complex would be formed between cytochalasin B and 4,6-O-ethylidene-D-glucose since there is no interaction between inward and outward facing sites. Le Fevre (1973) proposed the introverting hemiport model which is mechanistically similar to the lattice pore and tetramer models. This model accounts for the high Km for zero trans exit and equilibrium exchange, and for the low Km for infinite cis entry observed in the erythrocyte. However the introverting hemiport will show symmetrical rates of entry and exit, and does not account for the Vmax for zero trans entry being less than the Vmax for zero trans exit in the human erythrocyte. Giving this model symmetrical Km values to fit with the adipocyte

data leads to the hemiports becoming symmetrical microcarriers. The interaction of sugar molecules with the microcarriers on each face of the membrane should however be independent of one another so that this model is also inconsistent with the results of Gorga & Lienhard .

A model similar to the introverting hemiport has been proposed by Foley et al. (1980a) to account for hexose transport in adipocytes. The observation of different apparent Vmax values for 2-deoxy-D-glucose and 3-O-methyl-D-glucose suggests two different barriers for hexose entry (Foley et al. 1980a). Foley et al. propose that the accumulation of metabolites increases the Vmax of the rate limiting barrier for 2-deoxy-D-glucose. The overall Vmax of transport through the pore will be governed by the Vmax of the rate limiting microcarrier. Foley et al. (1980) point out that this model may have similar kinetics to the model of Regen & Tarpley (1974) of a carrier with an unstirred layer. A full kinetic analysis of the Foley et al. model has not been performed, although the unidirectional flux equation will be of a complex form containing 11 independent parameters. Holman et al. (1981) have derived an equation describing the inhibition of tracer exchange for this model and this equation predicts that different tracers may give different Ki's. As described previously there is no evidence for different Ki's when different tracers are used in the adipocyte (see p. 48).

A further development of two gate pore models is the allosteric pore (Holman, 1980) which accounts well for the asymmetry observed in human erythrocytes. Simulated results for hexose transport using this model are in close agreement with the observed results for the human erythrocyte hexose transport system. In particular this model accounts well for the low internal Km shown by infinite cis entry and infinite trans exit experiments (Holman, 1980). Further evidence for the involvement of

allosteric effects has been presented by Holman et al. (1981) who have reported negative cooperativity in human erythrocyte hexose transport (see p.186). Their results suggest that there is a shift from a low Km transporter form to a high Km transporter form as the concentration of hexose is increased. The allosteric pore model can also explain the observation by Levine et al. (1971) that the Ki for D-glucose inhibition of sorbose flux in the erythrocyte is much less than that predicted from the D-glucose exchange Km. The results of Holman et al. (1981) show that this anomaly is due to the Ki measurements being made using a low concentration of D-glucose whilst the exchange Km determinations are made using a high concentration of D-glucose. The experiments of Holman et al. (1981) used a range of D-glucose concentrations which revealed the low and high Km's for both experiments.

Kinetic analysis of the allosteric pore model gives seven independent parameters determining the rate of flux through the pore (Holman, 1980). The asymmetry and cooperativity of the allosteric pore model are dependent upon subunit interface stability constants. As described in the Introduction (see p. 52) the symmetry of transport is controlled by the subunit interface stability constants K_{BB} and K_{CC}. When $K_{BB} = K_{CC}$ symmetrical transport parameters will result. The parameters K_{BB} and K_{CC} also control the magnitude of the cooperative effects observed. If these parameters are less than unity the model will show negative cooperativity and if these parameters are greater than unity then the model will show positive cooperativity. No cooperativity will be observed in reciprocal plots if the V/K ratios for monovalently and divalently occupied pores are equal.

Thus the allosteric pore can be readily adapted to account for the observations of hexose transport in the adipocyte. There is little evidence for cooperative effects in the equilibrium exchange of 3-O-methyl-D-glucose in the adipocyte over a wide range of substrate concentrations (40μM to 60mM see p. 124). Since values of the $^{V}/_{K}$ ratios which are close to 1 give very small deviations from linearity in reciprocal plots cooperative effects may be present in the adipocyte, but may be undetectable by the present methodology.

This model is also consistent with the similar Ki's for the side specific analogues in the human erythrocyte and rat adipocyte (see p.204). The asymmetry of the erythrocyte is due to different subunit interface stabilities when the pore is divalently occupied from outside or inside. Negative cooperativity in the binding of a side specific analogue may lead to a non-linearity in $^{Vo}/V$ vs I plots. This may however be a small effect not detectable over the concentration ranges used.

The allosteric pore provides an obvious mechanism for the regulation of hexose transport by metabolites in the adipocyte. The interaction of metabolites with one of the subunits or a regulatory subunit could lead to an alteration in the transport parameters through allosteric effects.

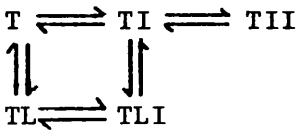
The adipocyte hexose transporter is relatively insensitive to inactivation by fluorodinitro benzene (FDNB)(Czech et al., 1978) when compared to the human erythrocyte (Bowyer & Widdas, 1958). The results of Holman et al. (1981) showed that the effects of FDNB were greater on the high Km component of hexose transport in human erythrocytes. This result was interpreted as an effect at the inner pore gate, which the

allosteric pore predicts to have a lower interface stability constant and therefore to be more reactive towards FDNB. The allosteric pore model proposes that the inner pore gate of the adipocyte is more stable, leading to symmetrical transport parameters, and is therefore less reactive to FDNB.

A major objection to the allosteric pore model as presently described seems to be that the model requires a stable ternary complex between 4,6-O-ethylidene-D-glucose and cytochalasin B which is inconsistent with the observations of Gorga & Lienhard (1981). Gorga & Lienhard have however interpreted their results using scheme (a).

(a)

(b)



Where T is the transporter, L the ligand (cytochalasin B) and I the inhibitor 4,6-O-ethylidene-glucose. Scheme (a) takes no account of the possibility of a stable ternary complex containing two 4,6-O-ethylidene-D-glucose molecules which competitively reduces the concentration of a stable 4,6-O-ethylidene-D-glucose/cytochalasin B complex (Scheme b).

Hexose transport in subcellular fractions of adipocytes

Martin & Carter (1970) reported that when plasma membranes were prepared from isolated adipocytes the D-glucose transport rate was 2-6 fold faster in plasma membranes prepared from insulin-stimulated cells when compared to plasma membranes prepared from basal cells. A similar five-fold increase in the rate of D-glucose transport was observed in the experiments with plasma membranes described in this thesis. This stimulation is considerably less than the stimulation of 3-O-methyl-D-glucose transport observed in intact adipocytes. The increased rate of transport in plasma membranes from insulin-treated cells is due to an increase in the Vmax for D-glucose transport (Ludvigsen & Jarett 1979, 1980). The Km for D-glucose transport was unchanged by insulin. Ludvigsen & Jarett showed that the Km for D-glucose transport was 9mM if membranes were prepared immediately after isolation of the cells. If however the cells were preincubated for 15 minutes before fractionation the Km was 26mM in membranes from both basal and insulin-stimulated cells. In intact cells the kinetic parameters for 3-O-methyl-D-glucose transport remain constant for several hours after isolation. In the experiments described in this thesis the Km for D-glucose transport in plasma membranes from insulin-treated cells was 15.6mM. This is higher than the Ki for D-glucose inhibition of D-allose exchange in intact cells (8.62mM). Some of these differences may reflect the difficulty of maintaining cell viability in the large-scale adipocyte preparations required for the preparation of isolated plasma membranes. Alternatively these effects may reflect the loss of a regulatory influence such as the effects of metabolites (see p. 195).

Studies on the microsomal fractions prepared from adipocytes have suggested the presence of facilitated diffusion systems for D-glucose (Suzuki & Kono, 1980, and Cushman & Wardzala, 1980). The results presented in this thesis show that D-glucose uptake into this fraction occurs rapidly via a non-specific route. This rapid non-specific process may well obscure a specific facilitated diffusion process for the transport of D-glucose into the vesicles. Carter et al. (1972) reported that the 'leakiness' of plasma membrane preparations could be reduced by brief sonication or exposure to hypotonic conditions. In the experiments described in this thesis sonication of the microsomal fraction was not carried out in order to maintain the orientation of the membranes.

Carter et al. reported that prior to sonication large amounts of the plasma membrane were present as sheets whereas after sonication the membrane was almost entirely vesicularised. Electron micrographs of the microsomal membranes (see p.117) indicate that the microsomal fraction contains many small closed vesicles consistent with the observation that D-glucose is taken up and trapped in a structure which is retained by the filter. It is interesting to note that phloretin apparently traps hexoses in the microsomal fraction during the 15 to 20 seconds required for the filtration. The use of reagents which alter the properties of the lipid bilayer (such as phloretin or anaesthetics) may provide a means of reducing the non-specific uptake of hexoses and allow further investigations of the transport of hexoses in the microsomal fraction. If hexose transport can be established the side specific analogues described in this thesis may allow a determination of the orientation of the transporter within the microsomal membranes.

The possibility that the microsomal hexose transporter exists in an inactive form has been suggested by the results of Karnielli et al. (1981). Their results show that the insulin mediated transfer of cytochalasin B binding sites from the microsomal fraction to the plasma membrane occurs slightly earlier than the increase in 3-O-methyl-D-glucose transport. Thus there is the possibility that the transporter is in some way inactivated or the active site is masked by an extrinsic protein when it is in the interior of the cell. Any form of masking would however have to be displaced by cytochalasin B or removed by detergent extraction in order to account for the results of Cushman & Wardzala (1980) and Suzuki & Kono (1980).

The substrate specificity of hexose transport in insulin-stimulated and basal adipocytes

The study of the substrate specificities of the hexose transporter in both basal and insulin-stimulated adipocytes presented in this thesis indicates that there is no significant change in either the hydrogen bonding or spatial requirements of the adipocyte hexose transport system on stimulation with insulin. This observation is consistent with the hypothesis of an increased number of sites of identical specificity in the plasma membrane as proposed by Wardzala et al. (1978). Changes in the transporter that would lead to a reduced turnover number might be expected to modify the spatial requirements for the transporter.

The effect of purines on the regulation of hexose transport in adipocytes

It has been suggested that adenosine and its nucleotides play an important role in the regulation of adipocyte metabolism (Dole, 1961 and Fain et al., 1972). The results of the experiments presented in this thesis show a very small effect of adenosine on basal cells with the maximal stimulation of 3-O-methyl-D-glucose transport occurring with 10μM adenosine. This effect (14.3% stimulation) is however considerably smaller than the 50% increase in the glucose oxidation rate observed by Taylor & Halperin (1976) with a similar concentration of adenosine. Experiments with exogenous adenosine deaminase and the adenosine transport inhibitor NBMI indicated that there is no effect of endogenous adenosine released by adipocyte preparations (Schwabe et al., 1973). The small effects of adenosine are difficult to study due to the instability of basal hexose transport in adipocytes. Other stimuli, especially mechanical agitation which are difficult to control reproducibly lead to large changes in the rate of hexose transport which obscure the small effect of adenosine.

In adipocytes maximally stimulated with insulin there was no effect of adenosine on the transport of 3-O-methyl-D-glucose. Adenosine did however appear to reduce the rate of hexose transport in adipocytes which had been stimulated with a sub-maximal dose of insulin. As described previously, maintaining sub-maximal effects is difficult, due to the rapid degradation of insulin, so this result should be interpreted with caution. The results of Taylor & Halperin (1976) showed a 200% increase in the rate of D-glucose oxidation when adenosine was added to cells maximally stimulated with insulin. This suggests that the effects of adenosine on insulin-stimulated cells may be on the enzymes of glucose

metabolism rather than an additional stimulation of the rate of hexose transport.

Of the purine and pyrimidine nucleosides tested inosine was found to give a small stimulation of hexose transport in basal adipocytes. Kypson & Hait (1976) reported that uridine and inosine were able to activate lipolysis in adipose tissue and increase the uptake of D-glucose by heart and skeletal muscle (Kypson & Hait, 1978). Kypson & Hait (1976) proposed that the effects of uridine were due to an increase in the glycogen content of the cells with no effect on the hexose transport rate, whereas inosine increased D-glucose uptake without affecting the glycogen content.

The stimulation of basal hexose transport by inosine is small when compared to the effects of insulin and it is therefore unlikely that inosine plays an important role in the action of insulin. The mechanism of inosine action is not clear. Malbon et al. (1978) have studied the binding of adenosine and adenosine analogues to the adipocyte plasma membrane and have reported two classes of adenosine binding sites unrelated to the adenosine transporter or adenosine deaminase. Malbon et al. found inosine to be a poor inhibitor of adenosine binding and it is therefore unlikely that the effects of inosine are mediated through purinergic receptors.

The only nucleotide to have any effect on the transport of 3-O-methyl-D-glucose was adenosine triphosphate, which inhibited hexose transport in insulin-treated cells. There was no effect of ATP in basal cells. Chang et al. (1974) reported similar results with adipocytes. Exogenous ATP has been shown to phosphorylate membrane proteins (Chang et al., 1974) but this phosphorylation was independent of insulin. Chang et al.

have suggested that these phosphorylations are not involved in the action of insulin but are involved in long-term regulation of hexose transport.

Possible second messengers for insulin action

A number of possible second messengers for insulin have been proposed (see Czech, 1977).

The regulation of hexose transport by cyclic nucleotides

The role of cyclic nucleotides as second messengers for the regulation of glycogen metabolism by catecholamine hormones is a well-established phenomenon. A number of reports have suggested that cyclic nucleotides are second messengers for the intracellular actions of insulin, and that they may also be involved in the regulation of hexose transport.

In the experiments described in this thesis a range of cyclic nucleotides and cyclic nucleotide derivatives were added exogenously to suspensions of adipocytes. No significant effects of cyclic nucleotides were observed, even over prolonged periods of up to two hours. Most of the reports describing the effects of cyclic nucleotides on hexose transport are based on experiments where the metabolism of D-glucose was studied and the results were interpreted as changes in the rate of glucose transport. These indirect experiments cannot differentiate between changes in the rate of transport of hexoses through the plasma membrane or changes in a rate limiting step in the metabolism of D-glucose.

Schimmel & Goodman (1971) reported that dibutyryl cyclic AMP increased the D-xylose space in whole adipose tissue and interpreted this result as an increase in the rate of hexose transport. The results presented in this thesis indicate that D-xylose is slowly metabolised by adipocytes. The long incubations (30 minutes) used by Schimmel & Goodman

would therefore have allowed metabolism of the radiolabelled D-xylose to occur. Thus the use of D-xylose does not give a true measure of the transport rate when long incubation times are used.

Correze et al. (1979) reported that low concentrations of dibutyryl cyclic AMP (0.1-0.5mM) stimulated the rate of 2-deoxy-D-glucose uptake in normal (basal) rat adipocytes. At higher concentrations of dibutyryl cyclic AMP (5-10mM) the uptake of 2-deoxy-D-glucose was inhibited. In their experiments Correze et al. used relatively long times for the uptake of 2-deoxy-D-glucose and report a small increase (40%) in the transport rate in response to a maximal dose of insulin. Foley et al. (1980b) have suggested that transport ceases to be the rate limiting step for 2-deoxy-D-glucose uptake after a few minutes and thereafter uptake is limited by the rate of phosphorylation. Thus the effects of dibutyryl cyclic AMP on 2-deoxy-D-glucose uptake reported by Correze et al. may reflect changes in the metabolic state of the adipocyte rather than changes in the rate of transport.

Insulin has been demonstrated to lower intracellular cyclic AMP levels by both inhibiting adenyl cyclase (Hepp & Renner, 1972) and by activating cyclic nucleotide phosphodiesterase (Kono et al.. 1976). In the experiments described in this thesis no inhibitory or potentiating effects of cyclic AMP or dibutyryl cyclic AMP were observed in insulin-stimulated cells. This result suggests that insulin mediated changes in cyclic AMP levels do not mediate the insulin stimulation of hexose transport.

Cyclic GMP

Cyclic GMP has also been proposed as an intracellular second messenger for insulin action by Illiano et al. (1973) who reported a four-fold increase in intracellular cyclic GMP levels in the presence of insulin. The results presented in this thesis show no effect of cyclic GMP or 8-bromo cyclic GMP on the rate of 3-O-methyl-D-glucose transport in basal or insulin-stimulated adipocytes. Pinkett & Perlman (1975) reported that exogenous 8-bromo-cyclic GMP stimulated the uptake of 2-deoxy-D-glucose in the rat diaphragm, although there was no effect of cyclic GMP or dibutyryl cyclic GMP. These experiments are subject to the criticisms described earlier regarding the use of D-xylose (see p.216) and 2-deoxy-D-glucose (see p.217) as a means of measuring hexose transport. It seems unlikely therefore that cyclic GMP is involved in mediating the action of insulin on the hexose transport system.

Another approach to elevating cyclic nucleotide levels inside the cell is the use of cyclic nucleotide phosphodiesterase inhibitors. The use of these compounds to study the effects of cyclic nucleotides on hexose transport is however complicated since these compounds may inhibit hexose transport. Theophylline and 3-isobutyl-1-methyl-xanthine have been shown to inhibit hexose transport in the human erythrocyte by Challis et al. (1980). The inhibitory effect of xanthine on 3-O-methyl-D-glucose transport in insulin-stimulated adipocytes may be related to the inhibition of hexose transport by methyl xanthines. If this is the case the stimulatory effect of xanthine on basal adipocytes may be underestimated, but will still be a small stimulation relative to that produced by insulin.

Overall the study of the effects of cyclic nucleotides presented in this thesis show that no major effects of these compounds occur when they are added exogenously to adipocytes. This suggests that cyclic nucleotides are not involved in the regulation of hexose transport by insulin.

Membrane ADP ribosylation

Malbon & Gill (1979) reported that cholera toxin stimulated the ADP-ribosylation of the adipocyte plasma membrane and led to an activation of lipolysis. Malbon & Gill showed that a number of membrane proteins were ADP-ribosylated including the regulatory subunit of adenyl cyclase. The results presented in this thesis show no effects of either substrates or inhibitors of ADP ribosylation on the rate of hexose transport or its stimulation by insulin in the adipocyte. NAD is the substrate for the enzyme responsible for ADP ribosylation (NAD-glycohydrolase). The lack of effect of NAD is unexpected since it is unlikely that this compound can penetrate the plasma membrane. Fain et al. (1972) have reported increases in the rate of lipolysis on the addition of NAD to adipocytes, and proposed that this occurred through the adenosine moiety. Thus the lack of effect of NAD on 3-O-methyl-D-glucose transport is consistent with the results discussed earlier which showed only slight stimulation of the hexose transport rate with purines.

Nicotinamides affect the synthesis of NAD (Clark et al., 1971) and therefore deplete the intracellular pool of NAD. In addition nicotinamides also inhibit NAD-glycohydrolase activity and would therefore markedly reduce ADP ribosylation (Purnell & Whish, 1980). A more specific inhibitor of ADP-ribosylation, 3-amino-benzamide, has been shown to enter cells and lead to changes in ADP-ribosylation in vivo. (Purnell, 1981). The lack of effect of these compounds on either basal or insulin-stimulated

adipocytes suggests that ADP ribosylation of membrane proteins is not involved in the regulation of hexose transport by insulin.

Anion transport

Beigelman & Hollander (1962) first demonstrated the ability of insulin to hyperpolarise the plasma membrane of adipocytes. In order to investigate the possible role of anion transport in the action of insulin the anion transport inhibitor SITS (Knauf & Rothstein, 1971) was added to adipocytes. There was no significant effect of SITS on basal or insulin-stimulated cells at a concentration which effectively blocks anion transport in erythrocytes (Knauf & Rothstein, 1971). This result does not rule out the possibility that cation gradients may be involved in the regulation of hexose transport (Clausen, 1975).

There is at present little evidence for the involvement of second messengers in the stimulation of hexose transport by insulin. The results presented in this thesis provide some further circumstantial evidence for the proposals of Cushman & Wardzala (1980) and Suzuki & Kono (1980) that insulin mediates its action through the recruitment of additional transporters from storage sites on the microsomal membranes.

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